Immune-mediated enteropathies

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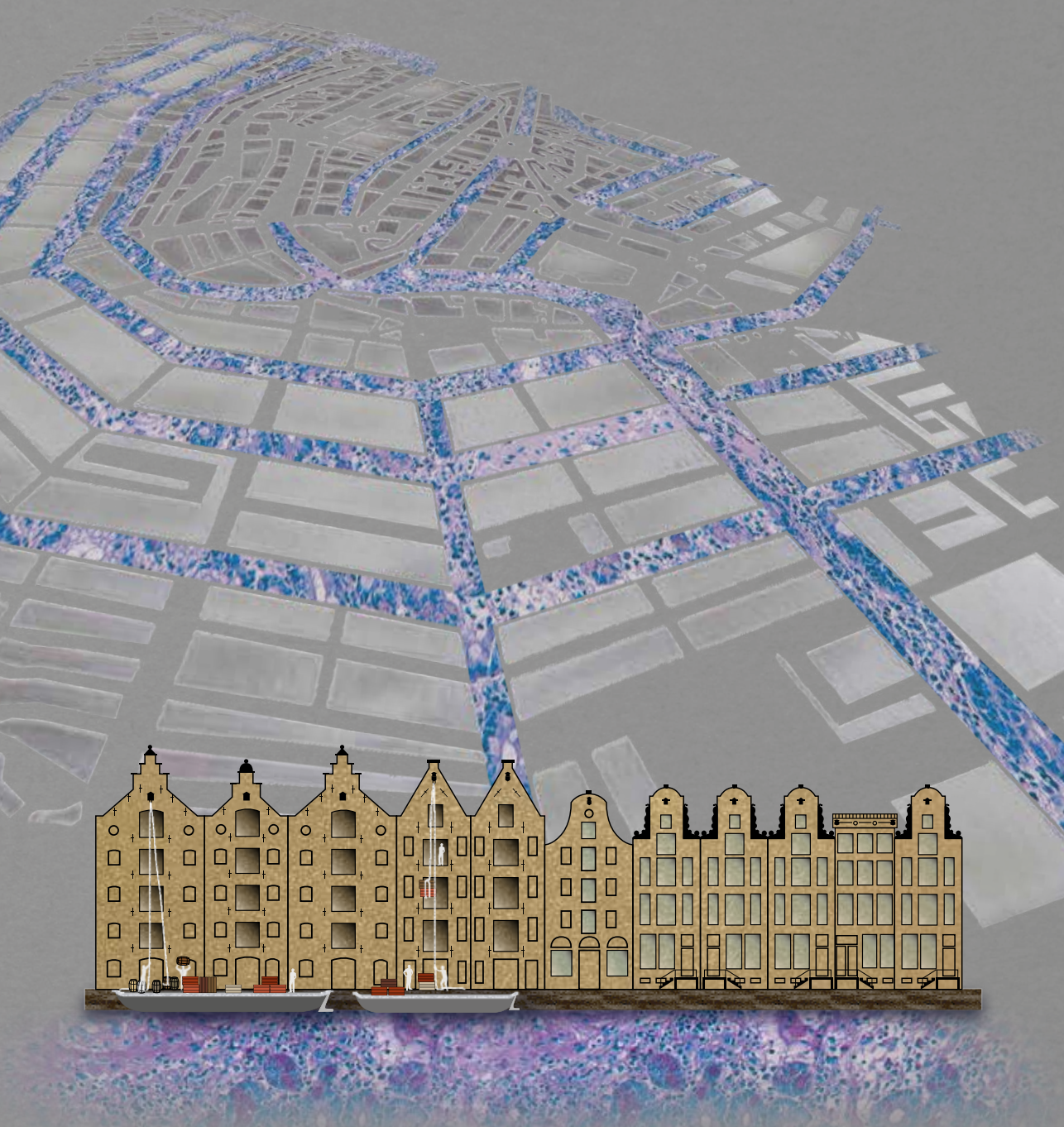
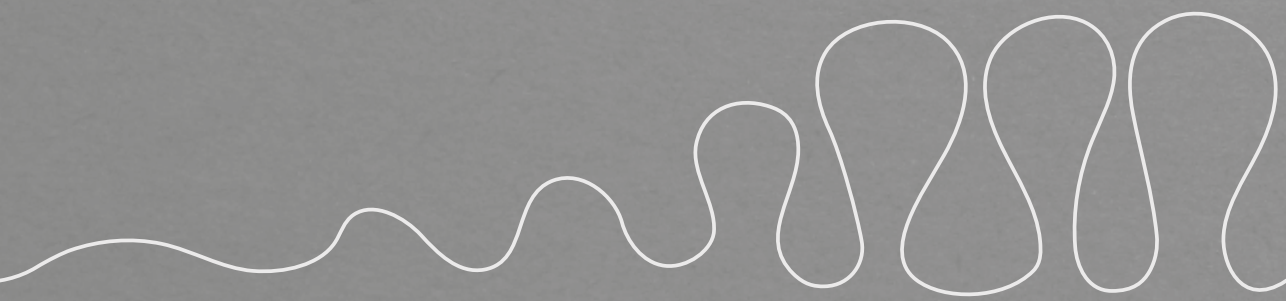
IMMUNE-MEDIATED ENTEROPATHIES

clinical, diagnostic and pathogenic insights



Roderik Leonardus Johannes van Wanrooij



IMMUNE-MEDIATED ENTEROPATHIES

clinical, diagnostic and pathogenic insights

Thesis: VU University Amsterdam

The work presented in this thesis was conducted at the Department of Gastroenterology and Hepatology, VU University Medical Centre, Amsterdam, The Netherlands.

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IMMUNE-MEDIATED ENTEROPATHIES

clinical, diagnostic and pathogenic insights

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ten overstaan van de promotiecommissie
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'It does not matter how slowly you go as long as you do not stop.'

Confucius

TABLE OF CONTENTS

SCOPE OF THE THESIS

PART IV SUMMARY, DISCUSSION AND FUTURE PERSPECTIVES

PART V ADDENDUM

SCOPE OF THE THESIS

Immune-mediated enteropathies encompass a variety of inflammatory diseases that affect the small intestine. Intestinal inflammation leads to destruction of the villi (villous atrophy), which drastically reduces the intestinal capacity to absorb nutrients and minerals. Patients with an (immune-mediated) enteropathy suffer from malabsorption-related symptoms such as chronic diarrhea, dehydration, weight loss and vitamin deficiencies, which in severe cases may lead to hospitalization for fluid resuscitation and total parenteral feeding. In a minority of severe cases the enteropathy may even lead to death.

The spectrum of immune-mediated diseases has been expanding over the last decade and some clinical, serological and histological features overlap between these enteropathies, and for this reason it can sometimes be challenging to differentiate between them. That being said, coeliac disease is still by far the most prevalent and well-known immune-mediated enteropathy. While the majority of patients with coeliac disease respond well to a gluten-free diet, symptoms and histological abnormalities persist or reoccur in a subgroup of patients despite strict dietary adherence. This is referred to as refractory coeliac disease. These patients are further divided based on the absence (type 1) or presence (type 2) of a premalignant intraepithelial lymphocyte population. This distinction is important since roughly half of patients with refractory coeliac disease type 2 develop an aggressive enteropathy-associated T-cell lymphoma that carries a dismal prognosis, while this is extremely rare in patients that lack a premalignant lymphocyte population (type 1).

The aim of this thesis was to gain insight in the clinical, diagnostic and pathogenic aspects of immune-mediated enteropathies, with a focus on (refractory) coeliac disease.

PART I provides an overview of immune-mediated enteropathies. In ***Chapter 1***, the introduction, current knowledge about clinical presentation, pathogenesis, histological and immunological features, as well as therapeutic strategies for immune-mediated enteropathies was reviewed. ***Chapter 2*** describes the outcomes of patients that were referred to a tertiary referral center for analysis of persisting symptoms despite a gluten-free diet. Also, a nation-wide survey was undertaken to determine the prevalence of refractory coeliac disease in the Netherlands. ***Chapter 3*** focusses on patients with adult-onset autoimmune enteropathy and reports on clinical, histological and immunophenotypical features, as well as their response to therapy. ***Chapter 4*** describes a patient with refractory coeliac disease type 1 with an abnormally large population of intraepithelial γδ T-cells that developed an enteropathy-associated T-cell lymphoma with unique phenotypical en genetic characteristics.

PART II contains three studies that aimed to improve the accuracy of diagnosing refractory coeliac disease. ***Chapter 5*** describes the diagnostic accuracy of immunohistochemistry and flow cytometry to identify the premalignant intraepithelial lymphocyte population characterized by an aberrant phenotype. In ***Chapter 6*** new serological markers were evaluated in their ability to differentiate between uncomplicated coeliac disease and complicated refrac-

tory coeliac disease. In ***Chapter 7*** it was examined whether antibodies against food antigen are useful as a serological marker for histological recovery in patients with refractory coeliac disease.

PART III focused on the pathogenesis of (refractory) coeliac disease. ***Chapter 8*** describes the studies on the origin and immunophenotype of the premalignant intraepithelial lymphocytes found in patients with refractory coeliac disease type 2. In ***Chapter 9*** intraepithelial lymphocytes from patients with coeliac disease and refractory coeliac disease type 1 and type 2 were cultured, and their cytokine production to various stimuli was measured with the aim to identify differences in the inflammatory response between these groups. The next two chapters studied the association of a polymorphism near the IL12A gene with coeliac disease, which thus far is one of the strongest non-HLA gene associations. IL-12 consists of multiple subunits and can form three different cytokines namely IL-12, IL-23 and IL-27, and these cytokines exert different effects in the immune system. ***Chapter 10*** provides an overview of associations with autoimmune diseases and IL12 related genes in order to gain insight in the role of the various IL12 gene polymorphisms. In ***Chapter 11*** the IL12A gene polymorphism associated with coeliac disease was studied with the aim to reveal the functional consequences of this genetic variant.

PART IV, the ***general discussion***, summarizes and discusses the main findings of this thesis, and shares recommendations for future research.

PART I

THE SPECTRUM OF IMMUNE-MEDIATED ENTEROPATHIES

Chapter 1

Immune-mediated enteropathies: from bench to bedside

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Manuscript submitted for publication.

ABSTRACT

Immune-mediated enteropathies are caused by excessive reactions of the intestinal immune system towards non-pathogenic molecules. Enteropathy leads to malabsorption-related symptoms such as (severe) chronic diarrhea, weight loss and vitamin deficiencies. Parenteral feeding and immunosuppressive therapy are needed in severe cases. Coeliac disease has long been recognized as the most common immune-mediated enteropathy in adults, but the spectrum of immune-mediated enteropathies has been expanding. Histological, serological and clinical features sometimes overlap between these enteropathies, and therefore it may be challenging to differentiate between them. Here, we provide an overview of immune-mediated enteropathies and discusses their pathogenesis, clinical presentation, diagnosis and treatment options.

INTRODUCTION

The small intestine is, in contrast to what its name suggests, characterized by a large surface that allows absorption of nutrients. Furthermore, it contains the largest part of the human immune system. The continuous exposure to the external environment necessitates the intestinal immune system to differentiate between innocuous and harmful antigens which leads to tolerance or the mounting of an immune response, respectively.[1] Excessive reactions of the intestinal immune system towards non-pathogenic molecules can lead to immune-mediated enteropathies (IME). Such a response may lead to destruction of mucosal surface which eventually results in the clinical signs of a malabsorption syndrome. Coeliac disease (CD) has long been recognized as the most common IME in adults.[2] While this still holds true, the spectrum of IME has been expanding over the last decades.[3] In addition to autoimmune enteropathy (AIE) and common variable immunodeficiency disease (CVID) as a cause of villus atrophy, perhaps the most important discovery has been the identification of olmesartan use as a cause of enteropathy.[4] Furthermore, indolent lymphoma of the gastro-intestinal tract often involves the small intestine and can be difficult to distinguish from other enteropathies.[5]

The main clinical problem in patients with IME is (severe) malabsorption and its associated complications. In addition, the increased risk of developing an aggressive enteropathy-associated T-cell lymphoma (EATL) observed in CD patients, CD patients refractory to a gluten-free diet (GFD) and in patients with AIE, even though it concerns a small absolute risk, has led to distress among patients and clinicians.

Differentiating between IME can be challenging as these diseases can partially overlap in clinical, serological and histological presentation. Recognition in an early phase is however important to adequately initiate appropriate treatment to prevent further disease, and possibly treatment related complications. In this review we provide a brief overview of the current knowledge regarding clinical presentation, establishing diagnosis, immunopathogenesis, and treatment options for the various forms of IME.

Coeliac disease

Coeliac disease (CD) is a gluten sensitive T-cell mediated enteropathy that occurs in genetically susceptible individuals. Gluten is the common denominator for staple proteins found in wheat, barley, and rye. They comprise hundreds of proteins with repeated sequences rich in proline and glutamine which renders them highly resistant to enzymatic breakdown.

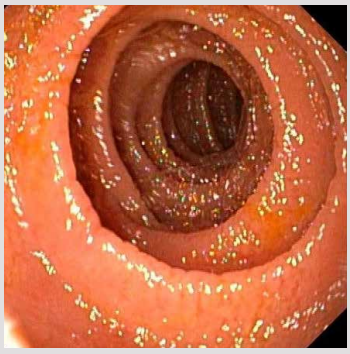
The estimated prevalence is 0.6% with a female to male ratio of 2:1.[6,7] CD does not only affect people of European descent, as originally thought. In fact, studies have shown rates of CD of up to 5.6% in specific North African populations.[8] The clinical presentation of patients with CD varies widely between asymptomatic to a severe malabsorption syndrome. While traditionally believed to be a disease of infancy, disease can occur at all ages and 40% of patients are being diagnosed above the age of 40 years.[9] The incidence has been steadily rising over the last 50 years, which can only partly be attributed to heightened awareness and better diagnostic modalities.[10]

The etiology is a complex interaction between genetic, environmental and immune factors that is not fully understood. Epidemiological studies have shown that first degree relatives have a 5-20% risk of disease and a high degree of concordance in monozygotic twins of approximately 70% points towards a strong genetic preponderance.[11] Positivity for the HLA class II alleles HLA-DQ2 or DQ8 is a prerequisite to develop disease but not sufficient since 30-40% of the Caucasian population is carrier of one or both of these alleles.[12] The genetic complexity is further underpinned by the identification of another 39 non-HLA loci many of which many are involved in the immune response.[13] Revealing the functional consequence of these genetic polymorphisms will provide further insight in the pathogenesis of this disease.[14] The HLA association in CD can be explained by the ability of HLA-DQ2 to bind the proline-rich gluten peptides that have survived gastrointestinal digestion. After intestinal absorption of gluten peptides the enzyme tissue transglutaminase is responsible for deamidation of these gluten peptides into negatively charged molecules. Deamidation is crucial in converting poorly immunogenic wild-type gluten peptides to highly immunogenic antigens for $CD4^+$ T-cells.[15] The peptides are then presented by HLA-DQ2 or DQ8 antigen presenting cells to gluten specific $CD4^+$ T-cells, which leads to activation of lamina propria T-cells that subsequently excrete proinflammatory cytokines such as interferon-γ (IFN-γ) and IL-21, the latter playing a role in T-cell dependent B-cell responses.[16] Interleukin 15 (IL-15) produced by epithelial cells stimulates expansion of intraepithelial lymphocytes (IELs) as well as upregulation of the activating NKG2D receptor which licenses these cytotoxic IELs to kill intestinal epithelial cells.[17] What drives the expression of IL-15 is an unanswered question, but may be related to epithelial stress triggered by microbiota-derived innate signals or by peptides present in wheat or gluten.[15] During disease activity numbers of both αβ T-cells and γδ T-cells are increased in the duodenal epithelium. In contrast to numbers of αβ T-cells that correlate with disease activity, the γδ T-cell population remains enlarged in all stages of the disease.[18,19] Interestingly, chronic inflammation permanently reconfigures the composition of tissue-resident γδ-IELs, yet the physiological relevance of these cells in CD pathogenesis is still unknown. Finally, $CD4^+$ T-cells induce B-cell differentiation into plasma cells that secrete anti-tissue transglutaminase IgA antibodies (TGA).

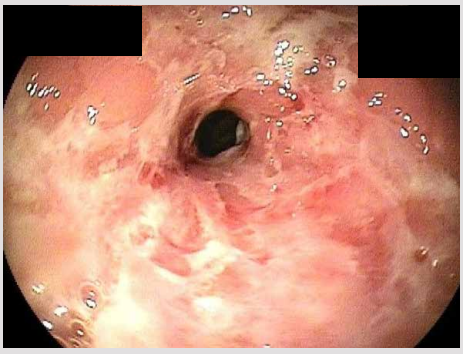
As such, CD has features of both a food intolerance and autoimmune disorder.

The events that culminate in the loss of tolerance to gluten in the small group of individuals that are HLA-DQ2 or DQ8 positive are poorly understood but changes in the microbiome, either caused by infections, antibiotic use or mode of delivery are under current scrutiny. The long-lasting hypothesis of a viral infection triggering the onset of disease is supported by murine studies showing that mice carrying HLA-DQ8, which develop gluten-specific $CD4^+$ T-cells, lose tolerance to gluten when infected by a reovirus.[20] Also recent epidemiological studies in children at risk of CD showed the appearance of transglutaminase antibodies within the three months following an episode of gastrointestinal infection whereas risk of CD decreased in children vaccinated against rotavirus before three months of age.[21]

For screening purposes serological markers such as TGA and anti-endomysium antibodies (EMA) are useful tests with a combined sensitivity and specificity of 98% and 100% respectively.[22] Yet, it should be kept in mind that 2%-3% of CD patients lack these antibodies.[23] Endoscopic abnormalities in CD include scalloping folds, reduced duodenal folds and mucosal cracks or a mosaic pattern (figure 1a).



a.



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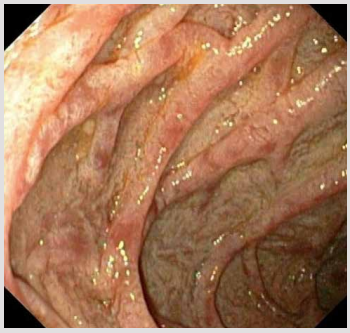
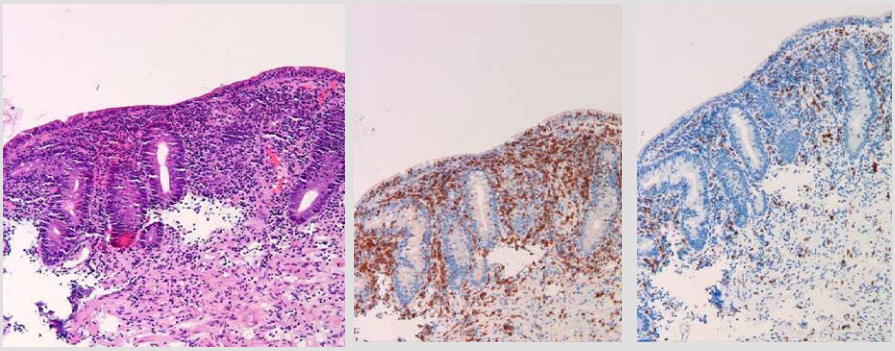


c.

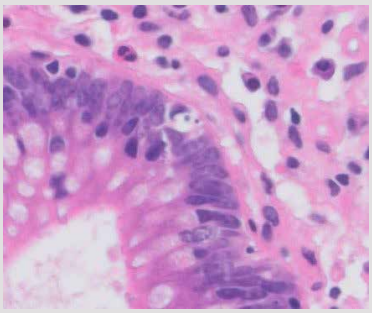
a. Duodenum in patient with coeliac disease shows mucosal cracks and scalloping folds.
b. Ulcerative jejunitis resulting in stenosis in a patient with refractory coeliac disease type 2.
c. Duodenum in patient with common variable immunodeficiency disorder showing an atrophic mucosa with mucosal cracks.

Figure 1 *Endoscopic findings in immune-mediated enteropathies.*

Nevertheless, these endoscopic findings lack sensitivity and specificity.[24] Histology will display intraepithelial lymphocytosis, crypt hyperplasia and villous atrophy to a various extent, which is defined accordingly to the modified Marsh criteria (figure 2a). [25]



a. *b.* *c.*



d.

a. Hematoxylin and eosin stain of duodenal biopsy shows complete villous atrophy, crypt hyperplasia and intraepithelial lymphocytosis (Marsh 3C) in a patient with (refractory) coeliac disease.

b.,c. Duodenal biopsies stained with CD3 (a, left) and CD8 (b, right) shows abundant expression of CD3 but not CD8 on intraepithelial lymphocytes, suggesting the presence of an (premalignant) aberrant lymphocyte population in accordance with refractory coeliac disease type 2.

d. Duodenum in patient with common variable immunodeficiency disorder showing an atrophic mucosa with mucosal cracks.

Figure 2 *Histologic findings in immune-mediated enteropathies.*

Intestinal TGA deposits measured in biopsy specimen appear more sensitive than serological test as these deposits can be found in seronegative CD patients.[26] These intestinal TGA deposits slowly decrease upon a GFD.[27] In addition, measuring TGA in culture supernatants of duodenal biopsies from CD patients appears even more sensitive and specific.[28] However, these techniques are laborious and costly and not used in daily clinical practice. A recently developed technique to detect gluten specific T-cells in biopsies and blood with the use of tetramer technology holds great promise since this technique is able to detect such cells even years after the instigation of a gluten-free diet.[29]

Refractory coeliac disease

After initiation of a GFD the majority of CD patients experience clinical improvement within weeks despite the fact that villous atrophy can persist over a long period (6 months to 5 years,

median of 1.3 years) in more than 40% of patients on a GFD.[30,31] Persisting or recurring symptoms are most often due to (inadvertent) gluten contamination.[32-37] Refractory coeliac disease (RCD) is defined as persisting symptoms and concomitant histologic abnormalities despite a strict gluten-free diet for at least 12 months as objectified by normalization of antibodies.[38] Extensive work-up may be necessary to eliminate other enteropathies which cannot be treated with a GFD.

RCD affects women two to three times more often than men, and usually affects individuals over 50 years of age.[39] RCD can be distinguished based on the absence (RCD type 1) or presence (RCD type 2) of a premalignant, clonal population of intraepithelial lymphocytes (IELs) with a distinctive phenotype.[40] Primary non-responsiveness to a GFD in newly diagnosed patients is observed in 30% of RCD type 1 and 50% of RCD type 2.[41-43] Population based studies show a low prevalence of RCD in CD patients, varying between 0.31%-0.70%.[39,44,45] This is in line with findings from tertiary referral centers that evaluate CD patients non-responsive to a GFD and only a minority of these patients (1-23%) are in fact diagnosed with RCD.[32-36]

The premalignant, clonal population of IELs in RCD comprise a lineage negative subset that is distinct from T, B, NK and lymphoid tissue inducer cells.[46] Lack of surface expression of CD3, CD8 and the T-cell receptor (TCR) in combination with intracellular expression of CD3 and surface expression of the natural killer (NK) receptor NKP46 separates these cells from normal IELs. Indeed these cells that are active in RCD patients on a GFD appear to be gliadin independent.[47] Furthermore, there is marked heterogeneity in cytokine responsiveness of the aberrant cell lines among RCD type 2 patients as most respond to IL-15, but others to IL-21R and or IL-2.[48]

In clinical practice three methods are used to identify this clonal aberrant cell population; TCR gamma (TCRG) clonality analysis and phenotypical analysis using immunohistochemistry or flow cytometry.[49] Common TCRG clonality analysis is however sensitive nor specific.[50,51] Additional analysis of the TCRB gene rearrangement pattern improves sensitivity, but is not yet widely available.[51,52] Immunohistochemistry uses the aberrant phenotype of these cells that loose both CD3 and CD8 on the cell surface, but still contain CD3 in the cell cytoplasm (figure 1b,c). Flow cytometric analysis allows a much more accurate identification of IEL subsets, including the aberrant IEL population, and is superior to immunohistochemistry, as well as TCRG clonality analysis in identifying pathologically enlarged IEL populations.[53-55]

Refractory coeliac disease type 1

RCD type 1 is defined by persistent villous atrophy despite a strict GFD longer than 1 year that leads to concomitant symptoms such as diarrhea, steatorrhea, abdominal pain, weight loss and-or nutritional deficiencies. Establishing the diagnosis of RCD type 1 can be difficult as a diagnostic test is lacking and the diagnosis is based on exclusion of other concomitant diseases that can result in similar symptoms.[56] Since a relatively high percentage of RCD type 1 patients was HLA-DQ2 and -DQ8 negative in some studies, it cannot be fully excluded that some of these patients may have suffered from other diseases than (R)CD.[57] Another challenge in the diagnosis of RCD type 1 is that persistent villous atrophy is frequently present in asymptomatic CD patients and those patients are referred to as slow responders and are generally believed not

to require additional immunosuppressive treatment. For instance, after one and five years of following a GFD respectively up to 80% and 40% of adult-onset CD patients still display villous atrophy.[58-64] So distinguishing patients with persistent villous atrophy without symptoms (slow responders) from those with symptoms (RCD type 1) might sometimes be controversial as for instance abdominal pain or diarrhea could well be caused by other concomitant disease such as irritable bowel disease. Not much research has addressed the immunopathogenesis of RCD type 1. It could be the result from minor gluten ingestion that maintains local inflammation without the emergence of CD-related antibodies in the serum. Alternatively, it has been proposed that inflammation in such patients is driven by a gluten independent autoimmune reaction.[26] This is still speculative and so far the IEL compartment in RCD type 1 and CD appears to be strikingly similar.[55] Furthermore, the cytokine profile in the serum of RCDI patients is comparable to that of CD patients, while again that of RCD type 2 patients is distinctive with upregulation of IL-6 and soluble Granzyme B.[65]

As both types of RCD patients lack CD-related antibodies other non-invasive markers correlating with mucosal recovery have been investigated. So far, intestinal fatty acid binding protein (i-FABP), bovine serum antibodies (BSA) and anti-*saccharomyces cerevisiae* antibodies (ASCA) showed a correlation with mucosal damage in RCD type2, but were not useful in clinical practice.[66,67]

While the 5-year survival in RCD type 1 patients is similar as compared to the general population, symptoms and-or nutritional deficiencies may warrant immunosuppressive treatment.[41,42] Budesonide, prednisone or a combination of prednisone with azathioprine are effective in inducing clinical and histological remission.[41-43,68-71] The risk of developing an EATL in RCD type 1 is low as these patients lack an premalignant aberrant IEL population.[72]

Refractory coeliac disease type 2

RCD type 2 is characterized by a clonally expanded IEL population with an aberrant (cytCD3$^+$ CD7$^+$ sCD3$^-$ CD4$^-$ CD8$^-$) phenotype.[40] In patients with an EATL the same T-cell clone has been found in both the aberrant IEL population and the EATL, which has led to the believe that aberrant IEL are precursor EATL cells.[73,74] RCD type 2 seems an inappropriate name as some patients presents with an aberrant IEL population at the time of CD diagnosis and therefore are strictly not yet refractory to a GFD.[75] During (capsule) endoscopy it is not uncommon to find an ulcerative jejunitis in these patients (figure 1b).

Aberrant clonal IELs derive from a small subset of unusual innate-like IELs present in the normal intestine.[46,51,76] The proliferation of these cells is gliadin-independent since dominant TCR clones in RCD type 2 patients show sequences distinctive from gliadin restricted T-cells.[47] Gluten specific CD4$^+$ T-cells isolated from CD duodenal biopsy specimens produce cytokines that are able to trigger proliferation of these aberrant IELs. In addition, IL-15 has an anti-apoptotic effect on aberrant IELs and somatic JAK1 or STAT3 gain-of-function mutations (or both), which confer hyper-responsiveness to IL-15, further augmenting the massive expansion of this cell population.[77,78] The expression of NKp46 in conjunction with IL-15 is believed to be responsible for enterocyte killing which may explain the severe ulceration often seen in these

patients.[76] RCD type 2 enterocytes also strongly express MICA, a ligand for NKG2D that is present on aberrant IELs which may further contribute to this uncontrolled inflammation.[49,79] In addition, a genetic association study (GWAS) identified a potential causal gene that may dysregulate the intestinal innate immune pathways in RCD type 2.[80]

Approximately half of the RCD type 2 patients develop an EATL within 5 years. [41-43] EATL are often located extra-intestinally which is in agreement with the finding that IELs are able to disseminate and can be found in the epithelium of the stomach and colon, as well as the bronchi and the skin.[81,82] Prognostic factors for the development of an EATL in a patient are the stage of cell maturation defined by the completion of the TCRB rearrangement, the scarcity of T-cell clones, as well as CD30 expression on aberrant IELs.[47,51,83]

The 5 year survival in RCD type 2 patients is poor (44-58%) which, in addition to severe malabsorption is mainly due to the development of an EATL.[41-43] There is a clear genetic association with both RCD type 2 and EATL with HLA-DQ2 homozygosity, suggesting a predisposing role of a strong anti-gluten responses.[49,57] Yet, there are conflicting results regarding the protective effect of a GFD on the development of EATL.[84-86] It should be noted that the majority of EATLs arise in non-recognized CD patients (primary EATL) instead of (R)CD patients (secondary EATL).[87] Currently it is unclear whether EATLs that develop from clonal aberrant IELs in RCD type 2 patients differ phenotypically or genetically from EATLs that develop in CD patients without these clonal aberrant IEL populations.

In attempt to eradicate the premalignant aberrant IEL population treatment various chemotherapeutic agents have been tried with limited success, probably because these cells have a low proliferative rate.[49] Cladribine, a purine analogue that induces T-cell depletion, has shown to decrease the risk of EATL development in RCD type 2 patients.[88] Cladribine is able to induce histological recovery but does not fully eradicate the aberrant IEL population.[80,81] Histological recovery appears the best prognostic factor for a decreased risk of EATL development in these patients, probably because it represents absence of ongoing inflammation.[88,89] In case of non-responsiveness to cladribine therapy, autologous stem cell transplantation has beneficial effects in patients that are eligible for this intense treatment.[90] A recent double-blind placebo controlled trial evaluating the effect of anti-IL-15 treatment did not find a difference between the groups in terms of the primary endpoint of aberrant intraepithelial lymphocyte reduction from baseline but appeared to have a positive impact on clinical symptoms.[91]

Autoimmune enteropathy

In the late 1970s an adolescent was described with diarrhea and autoantibodies binding to the cytoplasm of the intestinal epithelium, which responded to immunosuppressive therapy.[92] In the same period, familial syndromes were described with intractable diarrhea in the presence of other autoimmune disorders. In 1985 Unsworth and Walker-Smith were the first to use the term autoimmune enteropathy (AIE) for intractable diarrhea in the pediatric population, weight loss from malabsorption and immune-mediated intestinal damage in the absence of immunodeficiency.[93] Later the definition of AIE was has been refined to chronic diarrhea of more than 6 weeks with malabsorption, histological findings of partial or complete blunting of small intesti-

nal villi, increased apoptotic bodies, and deep crypt lymphocytosis with minimal intraepithelial lymphocytosis (figure 2d). The presence of circulating autoantibodies provides major diagnostic support, but its absence does not exclude AIE.[94] The incidence of AIE in children is low, in the order of magnitude of 0.1 per 100,000. The first diagnosed case of adult-onset AIE dates back to 1997 and while there are no epidemiological data available, the existence of less than 50 published cases suggests that it is even more rare than pediatric AIE.[95] AIE can occur in the context of identified genetic syndromes, e.g., the IPEX-syndrome (immune dysregulation, polyendocrinopathy, enteropathy, X-linked) caused by loss of function mutations in the FOXP3 gene on the X chromosome. The IPEX syndrome occurs only in males, whereas females are asymptomatic carriers. FOXP3 is widely expressed in $CD4^+/CD25^+$ subsets of T-cells and encodes for a transcription factor critical for the development and function of regulatory T-cells. In addition to the gastrointestinal manifestations, insulin-dependent diabetes mellitus, hemolytic anemia, and eczema are characteristic features whereas thrombocytopenia, hypothyroidism, and glomerulonephritis can also occur. Antibodies directed against a 75kDa antigen located in the gut and kidney epithelia are target autoantigens of enteropathy in IPEX syndrome.[96] This protein participates in interactions between membrane associated proteins and other cytoplasmic proteins involved in cytoskeletal arrangement, tight junction formation and in the regulation of paracellular enterocyte permeability and thus provide a plausible pathological rationale for disease. Although many patients succumb before the age of three due to either severe enteropathy or fatal infections unless they receive a hematopoietic stem cell transplant, some present with a milder form and survive to adolescence. The APECED syndrome (autoimmune phenomena, polyendocrinopathy, candidiasis, and ectodermal dystrophy), also known as autoimmune polyglandular syndrome 1 (APS-1), is another rare monogenic autoimmune disease, with an autosomal recessive inheritance. The responsible gene, AIRE (autoimmune regulator), encodes a protein involved in architectural organization of the thymic microenvironment essential normal processes of T-cell intra-thymic negative selection. Loss of function of this protein results in the development of autoreactive T-cells with clinical manifestations of Addison's disease, hypoparathyroidism, and hypogonadism, and chronic, recurrent mucocutaneous candidiasis in addition to severe GI disease. Anti-tryptophan hydroxylase-1 (TPH-1) antibodies are detected in 80% of APACED patients with gastrointestinal involvement and can be used to differentiate from the IPEX syndrome.[97]

The above prototypical examples demonstrate that disturbance of global immune regulation may cause an autoimmune reaction to the intestine. Increased levels of $CD4^+$ and $CD8^+$ T-lymphocytes in the lamina propria and intraepithelial presence of $CD8^+$ T-lymphocytes can be found in AIE yet the question as to how they result in tissue damage is not known. This could be the result of direct cytotoxicity against epithelial cells, through the secretion of cytokines or by antibody-dependent cellular cytotoxicity.[98] Mucosal $CD4^+$ T-cells from AIE patients secrete elevated levels of TNF-α,[94] and there have been some reports of successful treatment of adult-onset AIE with anti- TNF-α treatment which suggest that this proinflammatory cytokine plays a role in AIE.

In addition to AIE linked to syndromes such as IPEX and APECED syndrome, isolated

gastrointestinal forms of AIE with presence of anti-enterocyte antibodies (AEA)can occur in both children and adults. Although AIE is increasingly being recognized in adults, its characterization remains limited to a handful of case reports and small case series. Furthermore, its varied heterogeneous clinical associations and presentations suggest that adult AIE represents a collection of several entities unified by underlying immune dysregulation. From here we will focus on these adult forms of AIE.

Diarrhea in adult cases of AIE can be extremely voluminous and frequent. The diarrhea is persistent and non-bloody and is associated with significant weight loss and malabsorption. The duodenum is most commonly affected but involvement of stomach and colon have been described in 86% and 64% of cases, respectively.[99,100] Similar to the pediatric population, adults with AIE can also suffer from other autoimmune conditions such as rheumatoid arthritis and thymoma.[101,102]

Autoantibodies in AIE can be directed against the enterocyte (AEA) or goblet cells (AGA). In the former, they can target the intestinal brush border or the cytoplasm of enterocytes. In the largest case-series so far, involving 30 AIE patients, 50% displayed AEA, 13% displayed AGA while 37% were seronegative.[103] In the second largest case-series describing 13 patients, a higher percentage (86%) showed AEA antibodies, and in the other 15% AGA were detected (van Wanrooij et al, *submitted)*. AGA are generally considered nonspecific antibodies as they are also found in 28% of patient with CD or inflammatory bowel disease.[104,105] Whether AEA play a pathogenic role or also merely represent an epiphenomenon is still under debate. These autoantibodies seem to appear after the onset of disease, and therefore it has been hypothesized that AEA arise as a response to (auto)antigens that are released by damaged enterocyte. This idea finds support in the observation that low titers of AEA can also be found in other gastrointestinal disorders such as inflammatory bowel disease and cow's milk allergy.[106] Remarkably, as will be discussed below, olmesartan-induced enteropathy (OAE) is known to mimic AIE and AEA are found in up to 30% analyzed for this diagnosis.[107] So far, the brush border 75 kDa auto antigen is the only identified auto antigen specific for adult-onset AIE.[96] AIE-75 participates in tight-junction integrity and anti-AIE-75 antibodies are thought to hamper the intestinal permeability, which may precipitate inflammatory enteropathy.[100] AIE-75 is not a sensitive marker for adult-onset AIE, as AEA can be found in the brush border or the cell's cytoplasm, what strongly suggest the presence of other autoantigens (*van Wanrooij et al, submitted).* The histological presentation of autoimmune enteropathy is variable and shows an variety of presentations that often are indistinguishable from other inflammatory disorders. Originally partial or complete blunting of the villi, deep crypt lymphocytosis, increased crypt apoptosis, and minimal surface intraepithelial lymphocytosis, were described as histological patters in AIE. a new classification consisting of 4 types was proposed in 2017: (1) active chronic enteritis represents the most common form with a lymphoplasmacellulair infiltrate located in the lamina propria which can be accompanied with epithelial apoptosis and cryptitis (2) CD-like (3) graft-versus-host disease like, with prominent crypt epithelial apoptosis with a relative absence of inflammation(4) mixed/no predominant pattern.[108] The etiology and immunopathogenesis of adult-onset AIE is not known, but it is generally believed that immune

dysregulation, similar to that seen in the genetic syndromes in children, is responsible for the immunopathology in adult AIE. This is supported by the observation that in mouse models, blockade of PD-L1, the ligand of the immune checkpoint regulator PD-1, leads to the development of autoimmune enteritis, with T-cell infiltration, apoptosis of epithelial cells, and blunting of villi, which is reminiscent of that seen in human AIE.[109] Also, blockade of CTLA-4, a molecule expressed on, among others, regulatory T-cells may result in a pan-enteritis resembling AIE in melanoma patients treated with this drug.[110]

Due to the non-specific features of AIE, diagnosis is frequently delayed and patients may be initially misdiagnosed. The similarities with CD can be particularly challenging. Data regarding association with CD related HLA-DQ genotypes are conflicting. In one series, 23% (5/19) AIE patients carried one of the CD related genotypes which is comparable to the general population, while in our series this was much higher, namely 85% (11/13) (van Wanrooij et al *submitted*). Only two of these patients were diagnosed with CD, while CD was excluded in the other patients based on the lack of CD-related antibodies, normal γδ-IELs counts and most importantly, a lack of response to a GFD. Various treatment strategies in AIE have been reported including budesonide, thiopurines, cyclosporine, anti-tumor necrosis factor alpha, vedoluzimab and rituximab with at most were moderately effective.[94,100,101,111,112] Unfortunately, no therapeutic regimen has proven successful in all cases, and relapses frequently occur. Interestingly, according to one group of investigators, open capsule budesonide is effective in 85% of patients but these data need to be confirmed.[94,103] Most children with IPEX receive hematopoietic stem cell transplants and one adult patient with therapy refractory disease was successfully treated with autologous stem cell transplantation (van Wanrooij et al. *submitted*). So far, two patient with adult-onset AIE have developed an EATL. In comparison with EATL seen in CD this EATL displays a distinctive phenotypical and chromosomal abnormalities from an EATL seen in CD.[113,114]

Olmesartan-associated enteropathy

Only recently olmesartan, an angiotensin-II-receptor blocker used for treatment of hypertension, has been associated with a severe enteropathy.[4] Until now around 100 cases of olmesartan associated enteropathy (OAE) have been described.[115] Even though the overall risk of OAE in olmesartan users appears very low (<0.05%), it might be quite common in patients with unexplained enteropathy.[107,116]

Patients usually present with non-bloody diarrhea and weight loss, but severe dehydration with electrolyte imbalances and acute renal failure have been reported.[4] Men and women are equally affected usually in the seventh and eight decade of life, when the prevalence of hypertension is at its peak.[115] Some have reported a substantial concomitant presence of extra-intestinal autoimmune disease which indicates a genetic susceptibility.[107,116] This is supported by the increased percentage (64%) of patients that carry the HLA-DQ2 and-or HLA-DQ8 gene, in comparison to only 40% in the general population.[117] None of the cases reported so far presented with TGA or EMA. Further underlining a role for autoimmunity in OAE is the presence of circulating anti-nuclear antibodies (ANCA) that were observed in 80%

of patients and AEA in up to 30% of patients.[4,107,117] Histopathology can mimic AIE, especially with respect to intraepithelial lymphocytosis within or close to normal limits, loss of Paneth and goblet cells, and crypt apoptosis.[118] In 22% of cases subepithelial collagen deposition are reported that resemble collagenous sprue.[119] In addition to the small intestine other parts of the gastrointestinal tract are also commonly affected in OAE.[115] Lymphocytic and collagenous gastritis have been reported in 41% of patients, while microscopic colitis was identified in 27% of cases.[118] The mechanism by which olmesartan exerts its effect is not yet fully understood but several mechanisms have been proposed. A role for cell mediated immunity has been suggested based on the long delay between onset of olmesartan therapy and the development of diarrhea (and enteropathy).[4,120] Angiotensin-II-receptor blockers were initially thought to have inhibitory effects on transforming growth factor β (TGF β), a cytokine that plays a crucial role in gut immune regulation.[4] However, phosphorylation of smad2/3 was seen in duodenal biopsies from patients both on and off olmesartan indicating a functioning TGF β signaling pathway.[56] Involvement of the IL-15 signaling pathway has been suggested since IL-15R is upregulated on epithelial cells in the duodenum of OAE patients. Also, olmesartan is able to upregulate IL-15 and IL15R expression by Caco2 cells.[121] Another proposed mechanism by which olmesartan may exert its effect may be by disruption of Zonulin-1, a protein involved in tight junction formation.[121] Finally, *in vitro* studies have shown that angiotensin induces apoptosis of intestinal epithelial cells by acting on angiotensin-II-receptors.[122]

It is still under debate if OAE is drug-specific or whether it is a class effect. A nationwide study in France analyzing over 4,5 million patients starting treatment ARB's and angiotensin-converting enzyme inhibitor (ACEI) over a 5 year period for hospital admissions due severe malabsorption revealed a significantly increased risk for olmesartan, however failed to show in increased risk for any of the other sartans.[120] The selective role of olmesartan might be explained by its conversion into the active form in the intestine, its long half-life and its efficacy, on average a 30 fold higher than that of other sartans.[107] Sporadically, other sartans including telmisartan, valsartan, irbesartan en eprosartan have been associated with enteropathy, however, in these case-reports no re-challenge were performed.[114, 122-126]

Treatment of OAE by withholding administration of the drug is often sufficient to induce clinical and histological recovery. In severe cases, or patients with slow recovery, budesonide has been effective.[121,123] In some patients treatment with thiopurines, tacrolimus and anti-TNF also proved effective.[116]

Other medications associated with enteropathy

Ipilimumab is a monoclonal antibody against CTLA-4 that induces a T-cell response used for treatment of metastatic melanoma. The drug is well known for gastrointestinal side effects that includes enterocolitis which is probably initiated by T-cell activation in the gut.[124] Other immune checkpoint inhibitors such as anti-programmed death-1 antibodies (anti-PD-1, pembroluzimab, nevolimab) are associated with a similar risk of immune mediated entero-(colitis).[125] These immune checkpoint inhibitors are also able to induce an new onset of severe CD as was observed in a patient recently started on ipilimumab.[126] Development of CD has previously

been described in patients using interferon α and interferon beta 1, suggesting that these drugs contribute to CD development in genetically susceptible patients.[127,128] Mycophenolate mofetil is another drug that has been associated with new onset diarrhea and villous atrophy in four patients who used this drug to prevent rejection after renal transplantation, with complete recovery of symptoms after cessation of the drug.[129] More recently, watery diarrhea and apoptotic enteropathy has been associated with the use of methotrexate, capecitabine and TNF inhibitors, indicating that clinicians and pathologists should be aware of the expanding spectrum of drugs that can cause apoptotic enteropathy.[130]

Common variable immunodeficiency syndrome

Common variable immunodeficiency syndrome (CVID) is characterized by an impaired antibody response which leads to insufficient production of immunoglobulins. It is the most common symptomatic immune deficiency disorder with an estimated prevalence between 1: 25.000 and 50.000.[131] CVID results in recurrent infections as well as autoimmune complications. About half of the patients with CVID experience chronic diarrhea.[132,133] Often gastro-intestinal infections are the cause of diarrhea as a result of the defective immunoglobulin production present in these patients. Infection with *Giardia lamblia* leads to malabsorption as it leads to duodenal villous atrophy. Villous atrophy is commonly (31-50%) present in patients with CVID and gastrointestinal symptoms.[134,135] The exact mechanism for the development of villous atrophy in CVID is however unknown as eradiation of the pathogen does not always lead to mucosal and clinical recovery.[136] Impaired secretion of IgA (sIgA) has been proposed to be at least partly responsible as sIgA is a key player in regulating intestinal homeostasis.[135] SIgA prevents commensal bacteria in the small intestine from abundant contact with epithelial cells, and by doing so it might inhibit local production of nitric oxide which is an anti-microbiocide with a potent pro-inflammatory effect.[137] Furthermore, in mice deficient in polymeric Ig receptor that are unable to secrete IgA hyperplasia of IELs is observed, most likely as a compensatory defense mechanism.[138] This altered immune response may be more susceptible to dysregulation and contribute to intestinal inflammation seen in CVID patients.[135] Another interesting observation is that norovirus is an important pathogen for patients with CVID and a cause of CVID enteropathy. Analysis of duodenal biopsies revealed the presence of Norovirus in all 8 CVID patients with concomitant enteropathy and villous atrophy.[139] Viral clearance, either spontaneously or with the use of ribavarin, was associated with symptom resolution and histological recovery.[139]

Histological abnormalities in CVID encompass intraepithelial lymphocytosis, including influx of γδ-IELs.[135,140] For this reason, it is notoriously difficult to distinguish CD from CVID. Due to the impaired humoral immune response patients with CVID are commonly unable to produce antibodies of any kind, including CD-related antibodies. Yet, false positive CD-related antibodies can be found in the serum of CVID patients as a result of treatment with human immunoglobulins.[141] The histological hallmark of CVID is the absence or sparsity of plasma cells in the lamina propria.[135,141,142] This finding however does not correlate with the presence of villous CVID patients.[141] HLA-genotyping can be used to exclude CD, because HLA-DQ2 is not

overrepresented in CVID patients with villous atrophy.[143] If positive the only definite criterion to exclude CD is the lack of a histological response to a GFD.

The treatment of villous atrophy in the context of CVID can be complicated. Infection should be treated with the appropriate antibiotics or anti-parasitic therapy, often with a longer or more repeated course than normal. Chronic intestinal norovirus infection should be evaluated and treated when the patient is symptomatic. Replacement of human immunoglobulin is useful for pulmonary infections in patients with CVID, but appears to be of little use in clearing chronic intestinal infections.[3] This is most likely due to the fact that immunoglobulins are not delivered in the intestinal lumen. Budesonide is effective in inducing clinical remission in CVID patients with diarrhea and villous atrophy, but patients often remain dependent on steroids.[135] Anti-TNF alpha treatment or cyclosporine treatment was initiated in three patients without success.[135]

Indolent T-cell lymphoproliferative disease

Indolent T-cell lymphoproliferative disorders of the gastrointestinal tract are rare clonal T-cell diseases that more commonly occur in the intestines and have a protracted clinical course. In total 22 cases have been reported so far.[5,144-152] There is a strong male predominance, as 73% of the cases so far described were males. The median age is around 50 years, however cases in adolescents have also been described.[145,151] Lesions can occur anywhere in the gastrointestinal tract, with common involvement of the small intestine.[5]

The immunophenotype is heterogeneous, and can be either $CD4^+$, $CD8^+$, or $CD4^-/CD8^-$. Genetic alterations, including recurrent mutations and novel rearrangements, were identified in 8/10 (80%) lymphoproliferative disorders and involved frequent alterations of the JAK-STAT pathway genes.[152] These cases only sporadically transform into an aggressive lymphoma which underlines its indolent course. The diagnosis is challenging and such cases can be misinterpreted as IBD or an aggressive peripheral T-cell lymphoma.[5] Chemotherapy because of an initial diagnosis of peripheral T-cell lymphoma is generally ineffective. Watchful waiting is the preferred strategy in these patients. In a series of 10 patients with a median follow-up of 38 months (range, 9-175 months), 9 patients were alive with persistent disease and 1 was free of disease.[152]

Crohn's disease

Crohn's disease (CrD) is an inflammatory disease that can affect any part of the intestinal tract, with 80% of patients having involvement of the small intestine.[153] The most common site of small intestinal inflammation is the terminal ileum, while the proximal small intestine is affected in only 5% of CrD patients. Involvement of proximal small intestine is more often seen in pediatric patients.[154] Due to the transmural inflammation patients with CrD will often display symptoms resulting from intestinal ulceration and stenosis, as well as from fistulae or abscesses elsewhere in the abdomen or perianal region. The differential diagnosis of an ulcerative stenosis in the proximal jejunum is CrD, (complicated) CD or a malignancy such a lymphoma or adenocarcinoma. The dysregulated inflammatory response in CrD results from the interaction of genetic, immunological, microbial and environmental factors. Extensive efforts have been undertaken

to elucidate the genetic susceptibility in CrD, by means of genetic association studies involving large cohorts (>22.000 cases) that so far identified over 240 susceptibility loci.[155] The majority of these genes are involved in the immune system. In CrD both the innate as well as the adaptive immune system play a role. Defective first line (innate) defense against luminal bacteria and increased intestinal permeability leads to increased exposure of the mucosal immune system to these bacteria.[156] Dendritic cells and macrophages in CrD respond with production of pro-inflammatory cytokines TNF-α, IL-12 and IL-23. TNF-α prevents apoptosis of T-cells and secondarily increases the intestinal permeability.[157] IL-12 induces CD4 helper T-cell into a Th1 differentiation that produce IFN-y, IL-2 and IL-21. IL-23, which shares de IL-12p40 subunit with IL-12, induces a Th17 differentiation which leads to secretion of the pro-inflammatory Th17 cytokine thereby further enhancing inflammation.[158] ASCA have been used as a serological marker for CrD to differentiate it from UC, as it serves as a parameter for increased intestinal permeability.[159] Sensitivity and specificity are however limited.

Treatment consists of classical immunosuppressive drugs such as prednisone (systemic or topical), thiopurines as well as monoclonal antibodies that intervene in various parts of the immunopathogenesis. The first and most widely used MAB are anti-TNFα blockers. Other monoclonal antibodies that block the pro-inflammatory response are anti-IL12/IL23 blockers (ustikinimab) and JAK-inhibitors (tofacitinib). Furthermore, integrin-inhibitors (vedoluzimab) prevent migration of lymphocytes to the gut-associated lymphoid tissues.

Eosinophilic (gastro-)enteritis (EGE)

Eosinophilic gastrointestinal disorders are a group of diseases that include eosinophilic esophagitis, eosinophilic gastritis, eosinophilic gastroenteritis, eosinophilic enteritis, and eosinophilic colitis. Among these disorders, eosinophilic gastroenteritis is an uncommon and heterogeneous disease characterized by eosinophilic infiltration of the gastrointestinal tract in the absence of secondary causes. Patients can present with a variety of symptoms, depending on the site of inflammation and the depth of eosinophilic infiltration, which can be predominantly mucosal, muscular or serosal.[160] Current knowledge about EGE is based on case reports and small case series only.

EGE can occur in all age groups with a peak incidence between 30 and 40 years of age without a clear sex predisposition.[161] Genetic susceptibility is illustrated by the finding that 10% of EGE patients has a first degree relative with EGE.[162] Furthermore, a role for allergy is suggested as up to 75% of patients have an atopic medical history.[160]

Eosinophils are present in intestinal mucosa in the physiological state, but numbers within a healthy individual vary depending on the segment of the GI-tract.[163] For example, eosinophils are more abundant in the appendix and cecum. Furthermore, numbers of eosinophils vary among individuals depending on region, climate, season, age, infections and food allergens.[160]

Clinical symptoms depend on the layer of the intestinal wall that is involved.[164] When the mucosal and submucosal layer are predominantly involved, as is most frequently the case, patients present with abdominal pain, nausea, vomiting, diarrhea, malabsorption and weight loss. If the mucosal layer is predominantly inflamed this leads to bowel thickening and patients

can experience small bowel obstruction. Lastly, a serosal predominant form is recognized that is rare and can present with exudative ascites.[165]

Peripheral eosinophilia is not required for EGE diagnosis disease as it is often not present and it does not correlate with disease activity.[160] While a majority of patients has positive skin test responses, typical anaphylactic reactions are absent, which suggests a delayed-type of food hypersensitivity.[160] EGE patients display an upregulation of Th2 response, as illustrated by increased IL-4 and IL-5 production by peripheral blood T-cells, and IL-13 secretion by T-cells derived from the duodenal lamina propria after these cells were stimulation with milk proteins.[166,167]

A first step in treatment it is advocated to evaluate the presence of sensitization by food skin testing and-or specific IgE.[160] When sensitization is present a trial of food elimination can be initiated. When sensitization is not objectified or food elimination failed, in severe cases immunosuppressive drugs such as budesonide and thiopurines appear effective.[160]

Graft-versus-host disease

Graft-versus-host-disease (GVHD) located in the gastrointestinal tract is a common complication of hematopoietic stem cell transplantation but may occasionally occur after solid organ transplantation and blood transfusion.[168] Symptoms include nausea, vomiting and diarrhea and in more severe cases gastrointestinal bleeding, protein losing enteropathy and ileus. The histological abnormalities are classified into 4 grades: 1) isolated apoptotic epithelial cells without crypt damage 2) apoptosis with loss of individual crypts 3) apoptosis plus loss of 2 or more adjacent crypts 4) extensive crypt loss with mucosal denudation.[169] No significant influx of inflammatory cells is present in GVHD. GVHD is caused by allo-reactive donor T-cells and leads to secretion of the proinflammatory cytokines IL-2, IL-12, IFN-γ and TNF-α.[170] Damage to the mucosal barrier leads to translocation of microbial products and aggravates the inflammatory response.[170] In addition, Paneth cells responsible for production of antimicrobial peptides thereby regulating microbiota composition are targeted by GVHD.[170] The degree of Paneth cell loss is directly associated with the severity of intestinal GVDH, which has led to the belief that modulation of the microflora is a promising treatment strategy.[171]

The cornerstone of treatment currently consists of corticosteroid administration. In case of steroid-refractoriness various therapies including anti-T-cell antibodies (alemtuzumab, antithymocyte globulin), T-cell suppressive drugs (sirolimus, mycofenolate mofetil), and anti-cytokine biologicals (TNF-α, IL-6) have all shown disappointing effects.[172]

Table 1 *Characteristics of immune-mediated enteropathies.*

Enteropathy	Female %	Age years	HLA association	Autoanti-bodies	Histology	Treatment
Coeliac disease	70	All ages	>95% DQ 2.2 and-or DQ8	TGA, EMA	intraepithelial lymphocytosis, crypt hyperplasia, villous atrophy	glutenfree diet
Refractory coeliac disease type 1	60	40-70	>95% DQ 2.2 and-or DQ8	-	identical to coeliac disease	glutenfree diet, budesonide, tioguanin
Refractory coeliac disease type 2	70	40-70	40-50% DQ 2.2 homozygous	-	identical to coeliac disease but with $CD3^+CD8^-$ lymphocytes	glutenfree diet, budesonide, cladribin, stemcell transplantation
Autoimmune enteropathy	40-60	20-70	20-80% DQ2.2 heterozygous	AEA	4 subtypes: active chronic enteritis coeliac disease like graft-versus-host-disease like mixed type	budesonide, prednison, tioguanin
Olmesartan-associated enteropathy	50	50-90	70% DQ 2.2 heterozygous	AEA	loss of Paneth and goblet cells, apoptosis, often no intraepithelial lymphocytosis	discontinuation of drug, budesonide
Crohn's disease (located proximally)	50	10-40	-	ASCA	transmural inflammation, granuloma's	budesonide, prednison, thiopurines, anti-TNF, ustekinumab
Common variable immunodeficiency disorder	60	10-60	-	-	absence or scarcity of plasma cells	budesonide
Graf-versus-host disease	-	all ages	-	-	Apoptotic epithelial cells, possibly with crypt damage	prednison, anti-TNF
Eosinophilic enteritis	50	all ages, peak 30-40	-	-	Eosinophilic inflammation, most commonly in submucosa, alternatively in mucosa or serosa	food elimination, budesonide, thiopurines
Indolent T-cell lymphoma	30	50-60	-	-	Large population of $CD4^+$ or $CD8^+$ T-cells	budesonide

Abbreviations:
AEA anti-enterocyte antibodies;
ASCA anti-Saccharomyces cerevisiae antibodies;
EMA anti-endomysium antibodies;
HLA human leukocyte antigen;
TGA anti-tissue transglutaminase antibodies;
TNF tumour necrosis factor alpha.

CONCLUSION

The spectrum of immune mediated enteropathies has expanded, and differentiating between the various enteropathies can be challenging. In this review we have provided a brief overview of the current knowledge regarding clinical presentation, establishing diagnosis, immunopathogenesis, and treatment options.

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Chapter 2

Outcome of referrals for non-responsive coeliac disease in a tertiary center: low incidence of refractory coeliac disease in the Netherlands

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ABSTRACT

Objectives

Refractory coeliac disease (RCD) is a severe cause of non-responsive coeliac disease (CD) due to its association with the enteropathy-associated T-cell lymphoma (EATL). Conflicting data exist on the prevalence and the clinical manifestations of RCD type 1 and type 2. The aim of the current study was to provide insight in the incidence of RCD and in the distinction with other causes of non-responsive CD.

Methods

A total of 106 CD patients were referred to our tertiary referral center between January 2006 and December 2012 for evaluation of non-responsive CD. In addition, a questionnaire was sent to all 82 gastroenterology departments in the Netherlands to reveal whether a patient with RCD was currently being evaluated or had been treated between 2006-2012.

Results

During a six year period, a total of 31 patients were diagnosed with RCD (19 RCD type 1 and 12 RCD type 2). The nationwide survey revealed 5 additional patients with RCD type 1 and one patient with RCD type 2. This leads to an annual incidence of RCD of 0.83 / 10.000 CD patients. The remaining patients were diagnosed with involuntary gluten ingestion (21.7%), delayed mucosal recovery (11.3%), enteropathy-associated T-cell lymphoma (7.5%) and autoimmune enteropathy (1.8%).

Conclusion

This nationwide study reveals a low incidence of RCD in the Netherlands. Nevertheless, RCD is a clinically relevant disease entity in CD patients non-responsive to the gluten-free diet.

INTRODUCTION

A gluten-free diet (GFD) induces clinical improvement in the majority of coeliac disease (CD) patients within weeks to months.[1] Nevertheless, in a substantial group of patients longstanding mucosal abnormalities can be found despite a strict GFD. This can be either due to inadvertent gluten intake or slow mucosal recovery, i.e. lasting longer than 1 year.[2] The latter may occur in up to 80% of adult-onset CD patients, and this would decrease to a still considerable 40% after five years of treatment.[2-7] These patients should be distinguished from those who develop primary or secondary resistance to a gluten-free diet with persisting or recurring intestinal villous atrophy and symptoms of malabsorption. Patients with such refractory coeliac disease (RCD) can be distinguished based on the absence (RCD type 1) or presence (RCD type 2) of increased numbers (>20%) of intraepithelial lymphocytes (IELs) with an abnormal phenotype.[8,9] The latter are characterized by the absence of cell surface CD3 expression yet have CD3 contained within the cytoplasm (cyt$CD3^+$ s$CD3^-$ $CD45^+$ $CD7^+$ $CD4^-$ $CD8^-$ cells) and are considered lymphoma precursor cells.[10] Indeed, over 50% of patients with RCD type 2 develop overt lymphoma within 5 years.[11-13] Especially the distinction between RCD type 1 and slow response to a GFD can be a challenge in clinical practice.

RCD is considered a rare entity but the exact incidence and prevalence are not well known. Moreover, previous studies have shown discordant results regarding the distribution of the RCD subtype. Various reasons, including heterogenic definitions and diagnostic workup have been suggested to be responsible, at least in part, for these differences.[14] This distinction is however crucial, as RCD type 1 generally follows a benign course while RCD type 2 is associated with high morbidity and mortality.[15]

The aim of this study was (1) to provide insight in the prevalence of RCD in the Dutch population and (2) to gain insight in the underlying causes of persisting villous atrophy in patients where RCD has been excluded.

MATERIALS & METHODS

Patients

Patients included in this study visited the out-patient department of Gastroenterology at the VU University Medical Centre, Amsterdam, The Netherlands, for an one day diagnostic work-up for suspected complicated CD. Initial CD diagnosis was reassessed. Diet compliance was evaluated by a specialized dietitian and follow-up of anti-tissue transglutaminase antibody (TGA) and anti-endomysium antibody (EMA) titers. Furthermore, HLA genotyping, IgA serum levels, anti-enterocyte IgA and IgG antibodies, as well as hematological and biochemical parameters were determined. All patients underwent upper gastrointestinal endoscopy during which biopsies were collected from different locations in the duodenum. Four biopsies were scored according to the Marsh classification and evaluated for other causes of villous atrophy including giardiasis, collagenous sprue, eosinophilic duodenitis, absence of plasma cells and Whipple's disease. In addition, epithelial cell populations were evaluated as described below.

The diagnosis of RCD was based on persisting or recurring symptoms despite strict adherence to a gluten-free diet for at least 1 year and small intestinal villous atrophy in absence of other disease entities as mentioned above. To differentiate between RCD type 1 and RCD type 2 flow cytometric analysis of duodenal intraepithelial lymphocyte (IEL) subsets was used as it has shown to be the most accurate diagnostic tool currently availabel.[9,16] The diagnosis RCD type 2 was based on the clinically validated cutoff of more than 20% aberrant IELs.[9] CT-scan, videocapsule imaging, MRI-enteroclysis, colonoscopy and PET-scan were performed on indication.

According to the WHO-classification, enteropathy-associated T-cell lymphoma (EATL) was defined as a nonmonomorphic, pleomorphic, anaplastic, or immunoblastic tumor, with a $CD3^+$ $CD4^-$ $CD8^-$ $CD7^+$ $CD5^-CD56^-$ phenotype with expression of Granzyme B and TIA.[17] It should be noted that immunhistochemistry is unable to differentiate between surface and cytoplasmatic expression of CD3.[18] The CD3 expression by EATL cells is therefore thought to represent cytoplasmic CD3 expression.

Flow cytometric analysis of intraepithelial lymphocytes

Multiparameter flow cytometric immunophenotyping was performed on IEL suspensions, isolated as previously described.[9] Briefly, biopsies were vigorously shaken at 37 °C for 60 min in PBS supplemented with 1 mM DTT (Fluka BioChemika, Buchs Switzerland) and 1 mM EDTA (Merck, Darmstadt Germany). The released IELs were washed twice with PBS supplemented with 0.1% BSA (Roche Diagnostics) and subsequently stained for 30 min on ice, with fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridinin chlorophyll protein (PerCP) and allophycocyanin (APC)-labeled monoclonal antibodies directed against CD3, CD4, CD7, CD8, CD16 + 56, CD19, CD45 (all from BD Biosciences, San Jose, CA) and CD52 (Serotec, Düsseldorf, GermanyCD103 (IQ products, Groningen, Netherlands) Cytoplasmic staining of CD3 was performed after cell permeabilisation by Cytofix/CytoPerm Plus (BD Biosciences), according to the manufacturer's instructions. Stained cells were washed with PBS containing 0.1% bovine serum

albumin (BSA, Sigma) and analysed on a standard 4-color flowcytometer (FACSCalibur, BD Biosciences). The data were analysed using Cellquest software (BD Biosciences). Care was taken to analyse only viable cellular events based on light scatter properties.

Inventory of RCD type 2 prevalence in the Netherlands

To estimate the incidence of RCD in the Netherlands, all 82 gastroenterology departments in the Netherlands were sent a short questionnaire. The questionnaire included two questions: 1) Whether a patient with (suspected) RCD was currently being treated by any of the gastroenterologist practicing in that department. 2) Whether a patient with RCD had been diagnosed during the last six years by any of the gastroenterologist practicing in that department. Responses were provided per department. When no response was obtained after 14 days, one or more gastroenterologist per department received a phone consultation. In case a patient had been diagnosed with RCD further information was acquired to verify if the patient fulfilled the diagnostic criteria for RCD.[19]

Ethical approval

This study was in accordance with the ethical guidelines of our institution.

Statistical analysis

Incidence was reported as number per 100.000 inhabitants. 95% confidence intervals (CI) were calculated based on a Poisson distribution. IEL populations were reported as percentages of total, and per group as median and 10^{th}-90^{th} percentile. One-way ANOVA analysis of the data, for comparison between the groups, was performed using SSPS software (version 20, SPSS Inc., Chicago, Illinois). To correct for multiple testing, post hoc pair wise comparisons using Tukey's honestly significant difference test were carried out. A value of $p < 0.05$ was considered statistically significant.

RESULTS

Referrals

From January 2006 until December 2011 a total number of 106 patients were evaluated for suspected complicated CD. These patients were referred from 66 hospitals in the Netherlands. Clinical and demographic data are summarized in Table 1.

Table 1 *Patient characteristics.*

	CD patients with suspected RCD *n=106*	
Sex	Female: 56,8 %	
Age (median; range)	56.6 yrs (22 – 77)	
Time since onset of GFD	4 yrs (1 – 40)	
Symptoms		
• Absent		
• Present		
Symptomatic patients	*n=91*	
• Symptoms: persisting – recurring		44% - 56%
• Abdominal pain		28.6%
• Diarrhea only		15.4%
• Weight loss only		4.4%
• Diarrhea and weight loss		45.1%
• Fatigue		5.5%
• Fever / night sweats		0.9%
HLA-DQ genotype	*n=91*	
HLA-DQ2 heterozygous		56%
homozygous		26.4%
HLA-DQ8 heterozygous		8.8%
HLA-DQ2/DQ8 heterozygous		3.3%
HLA-DQ2/DQ8 negative		5.5%
Laboratory abnormalities		
• Anemia	*n=104*	46.2%
• Folic acid deficiency	*n=89*	6.7%
• Vitamin B 12 deficiency	*n=88*	8.0%
• Hypoalbuminemia	*n=100*	24.0%
Duodenal histology	*n=106*	
• Marsh 0		28.3%
• Marsh 1		14.2%
• Marsh 2		6.6%
• Marsh 3A		29.2%
• Marsh 3B		6.6%
• Marsh 3C		12.3%
• Ulcerative jejunitis		2.8%

Abbreviations: **CD** coeliac disease**;** **GFD** gluten-free diet; **RCD** refractory coeliac disease.

At time of referral the median age was 56.6 years (range: 22-77 years). The majority of patients (56%) presented with recurring symptoms, and had been on a GFD for a median of 4 years (range: 1-40 years) (Table 1). CD patients with persisting symptoms since diagnosis had been on a strict GFD for a median of 2 years (range: 1-5 years). The majority of patients (64.9%) presented with symptoms of diarrhea and-or weight loss. Anemia and hypoalbuminemia were present in 46.2% and 24% of patients, respectively (Table 1). Histological evaluation of duodenal specimens revealed villous atrophy (Marsh ≥ 3A) in 50.9% of patients. The remainder of patients did not fulfill the criteria for villous atrophy. Causes for their symptoms will be further discussed below.

Gluten contamination

Twenty-three (21.7%) CD patients were referred with persisting symptoms (Table 2). These symptoms were considered to be due to inadvertent gluten contamination as supported by positive serology and inadvertent gluten intake objectified by a specialized dietitian. In 20/23 (90.9%) of these patients histological evaluation of the duodenum was abnormal (Marsh ≥ 1). Two other symptomatic CD patients had no histological abnormalities yet positive serology and persistent gluten intake was substantiated by our dietitian.

Table 2 *Diagnosis of coeliac disease patients with suspected complicated coeliac disease.*

Diagnosis of coeliac disease patients suspected for complicated coeliac disease	n (%)
Gluten contamination	23 (21.7%)
Slow responders	12 (11.3%)
No duodenal abnormalities	33 (31.1%)
• microscopic colitis	3 (2.8%)
• inflammatory bowel disease	1 (0.9%)
• Helicobacter pylory infection	2 (1.8%)
• Irritable bowel syndrom	24 (22.6%)
• absence of CD	3 (2.8%)
Immunodeficiency disorder	1 (0.9%)
Autoimmune enteropathy	2 (1.8%)
RCD type 1	14 (13.2%)
RCD type 2	11 (10.4%)
CD with clonal γδ T-cells	2 (1.8%)
Secondary EATL	8 (7.5%)
Total	**n=106**

Abbreviations: **CD** coeliac disease**;**
EATL enteropathy-associated T-cell lymphoma;
RCD refractory coeliac disease.

Slow responders

Twelve patients were referred due to persisting mucosal abnormalities (≥ Marsh 3a) upon upper endoscopy despite the absence of clinical symptoms such as diarrhea, weight loss or abdominal discomfort (Table 2). These asymptomatic patients received follow-up endoscopy to confirm mucosal recovery, as recommended by local guidelines.[20] Median age of this group was 58 years (range: 34-77) and median time between index and follow-up endoscopy was 2 years (range 1-12). Eleven patients were HLA-DQ2 and/or DQ8 positive whereas one patient was homozygous for the HLA-DQ2 beta chain (*02). Serology was negative in all patients and dietary evaluation revealed no inadvertent gluten intake. In all these patients index and follow-up biopsies were re-evaluated by a specialized GI pathologist. Follow-up biopsies at our institution showed minimal abnormalities (Marsh score < 2) in 5 of these patients. In 7 patients persistent evident abnormalities (i.e., Marsh score > 2) were observed, but histological scores had improved compared to the index biopsies. Based on these findings these patients were diagnosed as 'slow responders '.

Suspected aberrant IEL populations

Three patients were referred with a supposed aberrant IEL population as diagnosed elsewhere with the use of immunohistochemistry. Two patients received follow-up endoscopy because they were experiencing symptoms: one patient reported fatigue and the other patient had unexplained recurrent fever episodes. The third patient was asymptomatic but received follow-up endoscopy in accordance with the previously mentioned guidelines. Flow cytometric analysis revealed normal IEL populations and histological examination no other abnormalities.

Non-responsive disease without duodenal abnormalities

In 33 symptomatic patients (31.1%) duodenal examination revealed no persisting abnormalities. Most reported symptoms included abdominal discomfort (42%), diarrhea (37%) and fatigue (11%).

In three patients the initial CD diagnosis was rejected based on absence of HLA-DQ2 or -DQ8 in combination with atypical histology and lack of CD-antibodies at time of diagnosis. In the other 30 patients the initial CD diagnosis could be confirmed. In two of these patients a *Helicobacter pylori* infection appeared to be the cause of their symptoms since symptoms disappeared after eradication. Nineteen of the remaining 28 patients underwent colonoscopy. Three patients were diagnosed with microscopic colitis, and one with inflammatory bowel disease. The rest of these patients were considered to suffer from CD-related irritable bowel syndrome and was treated accordingly.

Symptomatic patients with duodenal abnormalities on a strict GFD

In addition to the 12 patients that had been categorized as slow responder, 36 CD patients (34%) had evident duodenal abnormalities (Marsh ≥ 3A) despite being on a strict GFD, as indicated by the absence of CD-related antibodies and dietary evaluation (Table 2).

In eight patients a secondary EATL was present. Three patients suffered from concomitant

other disease entities: two were diagnosed with an autoimmune enteropathy, and another with common variable immunodeficiency disorder. Twenty-five patients (23.6%) were eventually diagnosed with refractory CD (RCD). Fourteen patients were diagnosed with RCD type 1. Opposed to patients in the slow responder group, these patients were experiencing malabsorption related symptoms and-or displayed iron- or vitamin deficiencies. Eleven patients were diagnosed with RCD type 2 based on the presence of increased numbers of aberrant T-cells. The median percentage of these cells was 59% (10th-90th percentile: 22-88) in RCD type 2 patients (Table 3). One RCD type 1 patient expressed an exceptionally high percentage (>70%) of monoclonal γδ T-cells in their epithelial layer, and was considered as a distinctive type of RCD, as more extensively described elsewhere.[21]

Table 3 *Intraepithelial lymphocyte phenotype per disease entity.*

	Active CD	CD in remission	RCD type 1	RCD type 2	EATL
CD3$^+$ T-cells					
• Median	98%	92%	97%	40%	44%
• 10th-90th percentile	84 - 99%	78 - 99%	69 - 99%	9 - 82%	15 - 98%
CD8$^+$ T-cells					
• Median	69%	74%	70%	19%	33%
• 10th-90th percentile	53 - 84%	38 - 86%	32 - 87%	4 - 55%	9 - 78%
CD4$^+$ T-cells					
• Median	5.5%	6%	2%	8%	7%
• 10th-90th percentile	2.5 - 20%	2 - 24%	0.5 - 12%	2 - 18%	4 - 10%
NK-cells					
• Median	1%	3%	2%	5%	3.5%
• 10th-90th percentile	0.1 - 5%	1 - 11%	0 - 5%	0.3 - 11%	1 - 7.8%
γδ T-cells					
• Median	26%	18%	22%	11%	10%
• 10th-90th percentile	13 - 52%	5 -49%	9 - 42%	0.4 - 20%	2 - 31%
B-cells					
• Median	0.1%	0.02%	0.1%	0.2%	0.2%
• 10th-90th percentile	0 - 1%	0 - 0.8%	0 - 0.9%	0 - 1%	0 - 1%
Aberrant IELs					
• Median	1%	4%	1%	59%	48%
• 10th-90th percentile	0.1 - 8%	0.6 - 12%	0.2 - 14%	22 - 88%	0.1 - 75%

Abbreviations: **CD** Coeliac disease; **RCD** Refractory coeliac disease; **EATL** Enteropathy-associated T-cell lymphoma.

Median of percentages of various cell subsets present in the duodenal epithelium.
[a] Significantly less CD3$^+$ T-cells in RCD and EATL as compared to all other groups p<0.001
[b] Significantly less γδ T-cells in RCD as compared to active CD p<0.01
[c] Significantly less CD3$^+$ T-cells in RCD and EATL as compared to all other groups p<0.001
[d] Significantly more aberrant T-cells in RCD and EATL as compared to all other groups p<0.001

Nationwide questionnaire: prevalence of RCD

In order to further define the prevalence of RCD in the Netherlands, all Gastroenterology departments were sent a questionnaire and 14 (17%) received a follow-up telephone call after two weeks. As a result, a response was received from all hospitals (100% response rate). Eight patients were reported to be diagnosed with RCD elsewhere. After careful evaluation of the patient history, six patients fulfilled the criteria for RCD. These included five RCD type 1 and one RCD type 2 patients. These patients continued their treatment at their own institution.

Over a six year period a total of 31 patients were diagnosed with RCD: 19 with RCD type 1 and 12 with RCD type 2. The annual incidence of RCD in the Dutch population (16.7 million inhabitants) is 0.031 per one 100.000 inhabitants (CI 0.022 – 0.044). According to a recent study using the Dutch Pathology Registry (PALGA) that has full nationwide coverage, the incidence of biopsy proven CD is 6.65 (CI 6.27 – 7.06) per 100.000 inhabitants.[22] This indicates that 1111 new patients were diagnosed with CD in the Netherlands in the year 2010, as compared to 5 patients with RCD (0.46%). Another study addressed the prevalence of recognized and unrecognized CD using serological markers and HLA-genotype in a study group representative for the Dutch population.[23] The prevalence of both recognized (0.016%) and unrecognized CD (0.35%) was 0.37% (CI 0.27% - 0.51%), which indicates that there are approximately 62.000 CD patients in the Netherlands. This indicates an annual incidence of RCD of 0.83 (CI 0.67 – 0.01) per 10.000 CD patients (both recognized and unrecognized).

DISCUSSION

Refractory coeliac disease is an extremely rare yet feared complication of CD. In our tertiary referral center, 23.6% of non-responsive CD patients were eventually diagnosed with RCD, which is higher than reported in other studies (0% to 10%).[24-27] This is most likely due to differences in the referral population. Inadvertent gluten contamination was observed in 21.7% of our referred patients, whereas this number was much higher in other studies, ranging between 35%- 45%.[24-27] In a substantial number of patients (21.7%) duodenal abnormalities were absent at time of RCD workup, an observation that is consonant with other studies[24-27] Autoimmune enteropathy (AIE) was diagnosed in two patients and in one patient a variable immunodeficiency disorder was identified. This reiterates the variety of sometimes rare causes that can underlie persistent villous atrophy and may mimic RCD.[28]

Despite the relatively high percentage of RCD in our referral population, our findings indicate that RCD is an extremely rare disorder with an annual incidence of 0.031 per 100.000 Dutch inhabitants and 8,4 in CD patients (both recognized and unrecognized). As far as we are aware other studies so far have reported on the prevalence, but not incidence, of RCD. This included three population based studies. In an unselected, population based cohort study from Derby, United Kingdom, five out of the 713 (0,7%) diagnosed CD patients fulfilled the criteria for RCD between 1978 en 2005.[29] In another population-based study encompassing 204 biopsy proven CD patients in Omsted County, United States, three patients were diagnosed with RCD over a 56 year time period.[19] A Finnish study reported the lowest prevalence (0.31%) of RCD in CD patients.[30] Finland has the highest prevalence (0.7%) of clinically diagnosed CD in a population, and has a high (88%) dietary adherence. Based on these observations, the authors suggested that early diagnosis and treatment of CD may result in a lower incidence of RCD. Other studies from tertiary referral centers have reported much higher prevalence's, ranging from 1.7%-10%.[7,19,27,31-33] However, these study populations might have been subject to selection bias, and are difficult to compare.

RCD type I appears to be more common than RCD type 2; in the published case series so far 56%-92 % of patients was diagnosed with RCD type 1 .[11-13,24,25,27,31,33-35] We have recently shown that for the diagnosis of RCD, immunohistochemistry may underestimate the number of aberrant T-cells in duodenal tissue and RCD type 2 patients with moderately increased numbers of aberrant IELs may erroneously be classified as RCD type 1.[16] This may also explain the variety in outcome in RCD type 1 patients between different centers.

CONCLUSION

In conclusion, this study underlines the wide variety of causes underlining non-responsive coeliac disease. This nationwide study on the prevalence of RCD shows that the incidence of RCD in the Netherlands, and in other European and North-American populations are more similar than previously thought. The likelihood of developing refractory disease is extremely low which may be reassuring and of help in the counseling of patients. Understanding why and identification of which patients may develop this severe complication of CD remains a major challenge. Collaboration between specialized centers to standardize diagnostic procedures and treatment protocols is therefore urgently needed.

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Chapter 3

Adult-onset autoimmune enteropathy: limited need for longterm immunosuppressive therapy

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ABSTRACT

Objectives

Adult-onset autoimmune enteropathie (AIE) is a rare cause of (severe) chronic diarrhea due small intestinal villous atrophy. We report on patients with adult-onset AIE in an European referral center.

Methods

Retrospective study including patients diagnosed with AIE in the Amsterdam UMC, location VUmc, between January 2003 and December 2019. Clinical, serological, and histological features and response to treatment were reported. The specificity of anti-enterocyte antibodies (AEA) was evaluated by examining the prevalence of AEA in (1) controls (n=30), and in patients with (2) coeliac disease (CD, n=52), (3) refractory coeliac disease type 2 (RCD type 2, n=18,) and (4) enteropathy-associated T-cell lymphoma (EATL, n=10).

Results

Thirteen AIE patients were included, 8 females (62%), median age of 52 years (range 23-73) and six (46%) with an autoimmune disease. AEA were observed in 11 cases (85%), but were also found in CD (7.7%), RCD type 2 (16.7%) and EATL (20%). Ten patients (77%) were HLA-DQ 2.5 heterozygous. Total parenteral nutrition was required in 8 cases (62%). Steroids induced clinical remission in 8 cases (62%). Step-up therapy with rituximab, cyclosporine, infliximab and cladribine in steroid-refractory patients was only moderately effective. Four patients died (31%), but four (31%) others are in long-term drug-free remission after receiving immunosuppressive treatment, including one patient who underwent autologous stem cell transplantation.

Conclusion

Adult-onset is a rare but severe enteropathy that occurs in patients susceptible for autoimmune disease. Immunosuppressive treatment leads to a long-lasting drug-free remission in a subset of patients.

INTRODUCTION

Autoimmune enteropathy (AIE) is a rare cause of chronic diarrhoea due to small intestinal villous atrophy. AIE was first identified in infants as hallmark of the Immune dysregulated Polyendocrinopathy Enteropathy X-linked (IPEX) syndrome that manifests at an early age and carries a poor prognosis.[1] Unsworth and Walker-Smith postulated the following criteria for AIE: (1) Severe malabsorption not responding to dietary restrictions (2) anti-enterocyte antibodies (AEA) and/or associated autoimmune conditions (3) and absence of immunodeficiency.[2] In 1997 it was recognized that AIE can also occur in adults.[3] Since then reports on adult-onset AIE comprised case-reports or small case-series with only one larger case-series by the Mayo Clinic that included 30 patients.[4] This group postulated refined criteria for adult AIE, implying that characteristic histological findings and absence of other causes for villous atrophy are additional major criteria for the diagnosis of AIE, while presence of gut-specific antibodies are not any longer required for the diagnosis.[5] Nevertheless, reported histological findings in AIE remained heterogenic and recently a subdivision has been proposed based on four histologic patterns.[6] The most common histologic pattern is that of an active chronic enteritis which is characterized by villous blunting with a mixed inflammatory infiltrate in the lamina propria, often with cryptitis and cryptabcesses but rarely with prominent epithelial apoptosis or intraepithelial lymphocytosis.[6] The coeliac disease-like pattern was observed in 20% of AIE patients while the remainder of patients displayed a graft-versus-host disease-like pattern or a mixed type pattern.[7] In clinical practice, the diagnosis of AIE therefore remains a challenge given the considerable overlap with other immune-mediated enteropathies such as coeliac disease, olmesartan-associated enteropathy, common variable immunodeficiency disorder (CVID) and refractory coeliac disease (RCD).[8]

Here we report on 13 patients diagnosed with adult onset AIE in a European referral centre and evaluate the value of AEA in the diagnosis of AIE.

METHODS

Patient selection and diagnostic workup

All patients diagnosed with AIE at the Amsterdam UMC, location VUmc, between January 2006 and December 2019 were included in this study. AIE was defined according to the previously described Akram criteria.[5] All patients presenting at our clinic underwent standardized diagnostic workup, as described in detail previously.[9] In short, medical history, symptoms and medication history were retrieved from the medical records. Recent use of angiotensin-II-inhibitors (e.g. olmesartan) and mycophenolate was an exclusion criterion. HLA genotype and serum auto-antibodies were determined (see below). Serum IgM, IgA, and IgG levels were measured. Duodenal biopsies were collected during upper endoscopy and histological features were evaluated by an experienced pathologist (EANB), and in addition phenotypical analysis of intraepithelial lymphocytes and T-cell receptor gamma (TCRG) gene rearrangement studies were performed. Colonoscopy, capsule endoscopy and abdominal imaging were performed on indication.

As control cohorts 30 healthy controls and patients with coeliac disease (n=52), RCD type 2 (n=18) or EATL (n=10) were included. AEA were determined at time of diagnosis of (R)CD or EATL. CD diagnosis was based on presence of TGA , Marsh>2 and a HLA-DQ 2.2, -DQ2.5 or -DQ8 genotype. RCD type 2 was defined as a lack of response to a strict GFD in CD patients with duodenal histologic abnormalities (Marsh III), and an increased percentage of IEL with an aberrant surface$CD3^-$ cytoplasmatic$CD3^+$ $CD7^+$ phenotype (>20% of total IELs).[10] Patients with EATL all presented with EATL and concomitant CD that was previously unrecognized.

Histological analysis

An expert pathologist evaluated all slides according to a standardized protocol. Biopsies were scored for the presence of inflammation (active and chronic), IEL count, villous atrophy, goblet cells, Paneth cells, apoptotic bodies and plasma cells (to evaluate CVID). Furthermore, histological patterns were classified as one of the four currently recognized histological phenotypes of AIE which include active chronic enteritis (type 1), CD-like (type 2), graft-versus-host disease-like (type 3), mixed type/no predominant pattern (type 4).[6]

Auto-antibody testing and HLA-genotyping

For HLA-genotyping whole blood was obtained for typing of HLA-DQA1* and DQB1* alleles, performed with a combined single-stranded conformation polymorphism (SSCP)/heteroduplex method by a semi-automated electrophoresis and gel staining method on the Phastsystem™ (AmershamPharmacia-Biotech, Sweden). For IgA and IgG anti-enterocyte antibody (AEA) testing both a direct and indirect immunofluorescence analysis using patient's serum on cryostat sections of normal human small bowel were performed. IgG against the AIE-associated antigen AIE-75 was tested by the radio-immunoprecipitation in the Volkmann laboratory, in Karlsruhe, Germany. Furthermore, antibodies against tissue transglutaminase (TGA), endomysium (EMA), goblet cell (AGCA), smooth muscle tissue (SMA), parietal cell (PCA),

neutrophil cytoplasmic antigen (ANCA), nuclear antigen (ANA), thyroid peroxidase (TPO) and thyroglobulin were determined, all using standardized serological techniques.

Intestinal fatty acid binding protein

Intestinal fatty acid binding protein (I-FAPB) serum levels of AIE patients were compared to levels reported in healthy controls and patients with RCD type 2 that we previously reported.[11] I-FABP levels were measured using a commercial ELISA kit (Hycult Biotech, USA) according to the manufacturer's specifications.

Phenotypical and TCRG gene clonality analysis of intraepithelial lymphocytes

IEL were isolated and analysed as previously described.[12] The epithelial layer was separated using DTT (Fluka BioChemika, Buchs Switzerland) and EDTA (Merck, Darmstadt Germany). Surface marker expression was evaluated using monoclonal antibodies directed against CD3, CD4, CD7, CD8, CD16+56, CD19, CD30, CD45, (all from BD Biosciences, San Jose, CA) and CD52 (Serotec, Düsseldorf, Germany). Cytoplasmic staining of CD3 was performed after cell permeabilisation by Cytofix/CytoPerm Plus (BD Biosciences). Stained cells were analysed on a standard 4-color flow cytometer (FACSCalibur, BD Biosciences). TCRG gene rearrangements were analysed in duplo on whole cryopreserved biopsy specimens. DNA was extracted using proteinase-K digestion and ethanol precipitation and subsequently analysed by multiplex polymerase chain reaction (PCR) amplification, using primers described by the BIOMED-2 consortium.

Treatment and follow-up

Per patient the treatment regime was noted and the patient was followed until December 2019 or death.

Statistical analyses

For comparing serum levels of I-FABP between patients with AIE, RCD type 2, EATL and healthy controls ANOVA (Krukal-Wallis) was performed, and a p-value <0.05 was considered statistically significant.

Ethical approval

The study protocol was approved by the Medical Ethics Committees from the Amsterdam UMC, location VUmc, Amsterdam, the Netherlands.

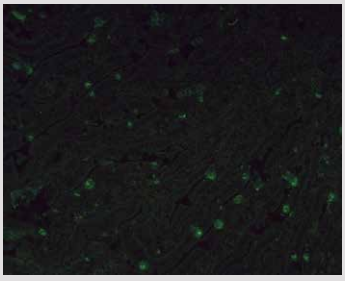
RESULTS

Patient characteristics at presentation

Thirteen patients fulfilled the criteria for the diagnosis AIE. All were Caucasians, eight were female (62%) and the median age at diagnosis was 52 years, with a wide range from 23 to 73 years (Table 1). All patients presented with profuse diarrhea (5-20 times per day) with a median time-interval of 3 months (range 1 month – 20 years) between onset of symptoms and time of diagnosis. Median stool production was 850 grams per 24 hours (range: 130 – 4000 gram) with significant loss of fat (median: 47 gram/24 hours; range 36 - 130 gram/24 hours). Diarrhea resulted in hypokalemia requiring intravenous suppletion in nine patients (69%). The median self-reported weight loss at time of AIE diagnosis was 15 kilograms (range: 3 -25 kg). Hypoalbuminemia was present in all patients with a median serum albumin concentration of 26 g/l (range: 13 g/l – 31 g/l). Abdominal discomfort was commonly reported (77%), but was consistently rated as mild.

Anti-enterocyte antibodies (AEA)

AEA were found in 10 out of 13 patients (77%): 4 were positive for both IgA and IgG antibodies, 4 were positive only for IgA antibodies, and 2 were positive only for IgG antibodies (Table 1). Previously, a 75 kDA protein (referred to as AIE-75) has been identified as an antigen for anti-enterocyte antibodies found in AIE patients.[13] AIE-75 participates in tight-junction integrity and anti-AIE-75 antibodies are thought to hamper the intestinal permeability, which may precipitate inflammatory enteropathy.[14,15] On indirect immunofluorescence AEA were found in the intestinal brush border (n=5), in the enterocyte's cytoplasm (n=2), or in both (n=3), see Figure 1.



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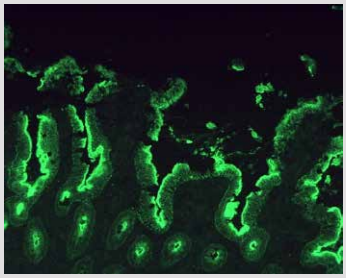

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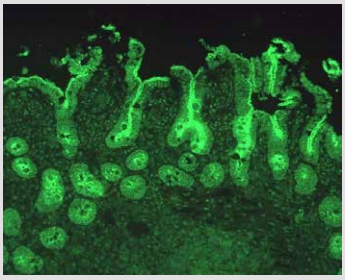

c.

*Immunofluorescence analysis of IgG anti-enterocyt antibodies (AEA) on normal humane small intestine. No expression is present in a healthy control **(a)**, while in **(b)** the intestinal brush border abundantly expresses IgG AEA, and in **(c)** IgA AEA is expressed in both the intestinal brush border as well as intracellular.*

Figure 1 *Indirect-immunofloresence for IgA and IgG anti-enterocyte antibodies.*

Table 1 *Patient characteristics.*

Case	Sex	Age	Time onset of symptoms to diagnosis	Presentation	Medical history	HLA-genotype	Gut-associated antibodies	AIE-75kd	Other autoimmune antibodies
1	F	23	<1 month	profuse diarrhea, weight loss	juvenile rheumathoid artritits	DQ2.5 heterozygous	IgA, IgG (BB) AEA	+	-
2	F	63	<1 month	profuse diarrhea, weight loss	-	DQ2-8 negative	IgA (Cyt) AEA	-	SMA, PCA
3	F	36	7 months	profuse diarrhea, weight loss	COPD, deep venous thrombosis	DQ2.5 heterozygous	IgA (BB, Cyt) AEA	-	ANA
4	F	40	4 months	profuse diarrhea, weight loss	type 2 diabetes mellitus	DQ2.5 heterozygous	-	n.p.	TPO
5	M	67	6 months	profuse diarrhea, weight loss	hypothyroidism	DQ2.5 heterozygous	IgA (BB) AEA	-	PCA, TGab
6	M	52	2,5 years	profuse diarrhea, weight loss	-	DQ2.5 heterozygous	IgA, IgG (BB) AEA	-	SMA
7	M	57	3 months	profuse diarrhea, weight loss	-	DQ2-8 negative	IgG (BB) AEA	+	ANA, pANCA
8	M	73	2 years	weight loss	appendectomy	DQ8 heterozygous	IgG (BB, Cyt) AEA	-	ANA, pANCA
9	M	59	2 months	profuse diarrhea, weight loss	coeliac disease	DQ2.5/8 heterozygous	IgA (Cyt) AEA	-	PCA
10	F	30	3 months	profuse diarrhea, weight loss	coeliac disease	DQ2.5 heterozygous	IgA, IgG (BB, Cyt) AEA	+	ANA, SMA
11	F	52	2 years	profuse diarrhea, weight loss	Larsen syndrome, appendectomy,	DQ8 heterozygous	IgA AGA	n.p.	-
12	F	36	20 years	profuse diarrhea	Type 1 diabetes mellitus, cholecystec-tomy, atrofische gastritis	DQ2.5 heterozygous	IgA AGA	n.p.	PCA
13	F	39	<1 month	profuse diarrhea, weight loss	hypothyroidism, asthma, chronic myelocytic leukemia	DQ2.5 heterozygous	IgA, IgG (BB) AEA	+	PCA

Abbreviations:

AEA anti-enterocyte antibodies;
AGA anti-goblet cell antibodies;
AIE autoimmune enteropathy;
AIE-75 autoimmune enteropathy related 75-kilodalton antigen;
ANA antinuclear antibody;
BB brush border;
Cyt cytoplasmic;
F female;
HLA human leukocyte antigen;
M male;
pANCA perinuclear anti-neutrophil cytoplasmic antibodies;
PCA anti-parietal cell antibodies;
SMA anti-smooth muscle antibodies;
TPO anti-thyroid peroxidase antibodies;
TGab anti-thyroglobulin antibodies.

In our cohort, anti-AIE-75 antibodies were found in 4 out of 10 (40%) patients tested, and in these patients AEA were all located in the brush border. In the other 60% the specific autoantigen was unidentified. There was no correlation between the AEA titer nor the location of the AEA (brush border vs cytoplasm) and the severity of histological inflammation or villous atrophy, but numbers are likely too small to detect such a correlation. After onset of treatment AEA titers often decreased or became negative yet this appears independent of clinical or histologic responses. Two patients with histological abnormalities corresponding with AIE lacked anti-enterocyte antibodies but instead anti-goblet cell antibodies (AGCA) were found. None of the patients with AEA additionally displayed AGCA. In one case (case 5) with duodenal histologic abnormalities concomitant with AIE neither AEA nor AGCA were found at all.

With the goal to evaluate the clinical specificity of AEA we performed additional tests in healthy controls and patients with uncomplicated (CD) and complicated coeliac disease (RCD type 2, EATL). AEA were absent in 30 healthy controls (0%), but could be detected in patients with CD 4 / 52 (7.7%), type 2 RCD 3 / 18 (16.7%), and EATL 2 / 10 (20%).

Intestinal fatty acid binding protein

Intestinal fatty acid binding protein (I-FABP) is a protein present in the cytosol of enterocytes and is released into the serum after intestinal damage. Indeed, I-FABP serum levels correlated with the severity of intestinal damage in CD patients.[16] With the aim to compare the extent of intestinal damage, serum I-FBAP levels in the group of AIE patients were measured and compared to the levels that we have previously reported in healthy controls and RCD type 2 (Table 3).[11] I-FABP levels in the serum of AIE patients were significantly higher [1150 (45 – 2650) pg/ml: $p<0.05$] than the levels in healthy controls [229 (85 – 1338) pg/ml], but similar to those in RCD type 2 patients [870 (106 – 2234) pg/ml].[11]

Duodenal histology

Total villous atrophy was seen in in 11 / 13 cases (Table 2). Six of those 11 cases showed clear intra-epithelial lymphocytosis (>40 IELs/ 100 enterocytes), whereas in four a mild increase of IELs (> 30 < 40 IELs per 100 enterocytes) was found. In only one out of eleven cases (case 8) no intraepithelial lymphocytosis was seen. In this group with total villous atrophy eight cases showed active and chronic inflammation, whereas in three only chronic inflammation was observed. In 6 cases goblet cells were reduced or absent, of whom in four Paneth cells were affected as well. On the contrary, Paneth cell depletion always co-occurred with goblet cell depletion. Furthermore, apoptotic bodies were found in 8 cases, including 3 cases with normal goblet cells and Paneth cells. Vice versa, 2 cases presented with impaired numbers of goblet cells but without apoptotic bodies.

Remarkably, in two cases (case 5 and 12) presenting with severe steatorrhea, weight loss and AEA or AGCA, no villous atrophy could be observed in the duodenal biopsies. Nonetheless, other histologic features of AIE were present in these patients: in case 12 apoptotic bodies were found in absence of goblet- and Paneth cells, while case 5 showed active and chronic enteritis. Also, I-FABP levels in these patients were elevated implying pathological enterocyte damage.

Both patients responded to immunosuppressive treatment which further supports the notion that the enteropathy was indeed immune mediated.

Using the histology based classification, the majority of patients (8/13 62%) was classified as active chronic enteritis (type 1), 3 (23%) as CD-like (type 2), and two (15%) as GvHD-like (type 3).[6]

Table 2 *Histological findings in patients with AIE.*

Case	villous atrophy	IEL count / 100 entero-cytes	Goblet cells	Paneth cells	apoptotic bodies	inflamma-tion	Histological classifica-tion	stomach	colon
1	complete	>40	-	0	0	active & chronic	Type 1	0	
2	complete	>40	0	0	++	chronic:	Type 2	0	
3	complete	>40	- -	- -	0	active & chronic	Type 1	0	chronic active colitis
4	complete	>40/	- -	-	++	active & chronic	Type 1	0	
5	none	<30	0	0	0	active & chronic	Type 1	0	
6	complete	>30 <40	-	0	++	active & chronic	Type 1	0	
7	complete	>30 <40	- -	-	++	active & chronic	Type 1	0	
8	complete	<30	0	0	+	chronic	Type 3	0	
9	complete	>40	0	0	++	active & chronic	Type 1	0	
10	complete	>40	0	0	++	active & chronic	Type 1	0	
11	complete	>30 <40	- -	-	+	chronic	Type 2	AIG	chronic active colitis
12	none	<30	- -	--	+	none	Type 3	AIG	
13	complete	>30<40	0	0	0	chronic	Type 2	FLG	

++ severe increase, + modest increase, 0 normal, - modest decrease , -- absent

Abbreviations: **AIG** autoimmune gastritis;
FLG focal lymphocytic gastritis.

Histological classification of AIE according to Umetsu and collegues[6]

- type 1: active chronic enteritis,
- type 2: coeliac disease-like,
- type 3: graft-versus-host-disease-like,
- type 4: mixed/no predominant pattern.

Phenotype of intraepithelial lymphocytes

The epithelial T-cell infiltrate was dominated by $CD8^+$ IELs over $CD4^+$ IELs in 11 out of 13 patients, which is also seen in normal duodenum and in CD. In the two other patients the $CD4^+$ T-cell population was slightly larger than the $CD8^+$ T-cell population (CD4/CD8 ratio 1.1 to 2.8). The γδ-IEL population is commonly increased in CD, with the γδ-IELs making up over 14% of total IELs in the majority of CD patients, but in none of the AIE patients, including the two patients who were previously diagnosed with CD, was such an upregulation noted.[17] Another relevant IEL subset are those IELs with an aberrant phenotype ($surfaceCD3^+$ $cytoplasmaticCD3^-$ $CD7^-$ $CD45^+$) considered a premalignant cell population and characteristic for RCD type 2, but again no significant expansion was observed in any of the AIE patients.[18]

Table 3 *Intraepithelial lymphocyte populations in AIE.*

case	Intraepithelial lymphocyte phenotype							TCRG clonality	I-FABP pg/ml
	%CD3	%CD8	%CD4	%NK-cell	% B-cell	% γδ T-cell	%CD3		
1	83	64	24	6	1	6	3	polyclonal	45
2	92	31	56	5	1	10	4	polyclonal	95
3	95	89	8	3	0	1	3	monoclonal	1720
4	98	85	14	1	1	7	1	polyclonal	2200
5	67	56	9	11	0	3	12	monoclonal	1150
6	95	87	8	0	4	0	0	polyclonal	1250
7	95	83	11	2	2	1	1	polyclonal	947
8	97	60	40	1	2	1	1	polyclonal	1030
9	69	31	38	26	2	1	5	monoclonal	1760
10	93	83	6	5	2	9	5	monoclonal	126
11	98	85	15	1	0	1	2	polyclonal	563
12	94	82	12	1	0	0	0	polyclonal	2650
13	98	85	14	1	1	7	1	monoclonal	2490

TCR gamma (TCRG) gene clonality analysis

TCRG gene rearrangement studies were performed in all 13 patients. In five (38%) patients a monoclonal rearrangement pattern was found, while the pattern was polyclonal in the other eight (62%) patients.

Involvement of the gastrointestinal tract

Extent of involvement of the gastrointestinal tract was evaluated in all patients by gastroduodenoscopy and colonoscopy while seven patients also underwent capsule endoscopy of the small intestine. No macroscopic abnormalities were observed in the stomach in 10 patients, yet histologically a nonspecific chronic inflammation was present in all cases. In addition, in two patients histological signs of autoimmune gastritis (AIG) with congruent parietal cell antibodies (PCA) were found and in one patient focal lymphocytic gastritis. All patients had involvement

of the proximal intestine whereas the distal part was involved in 6 out of 13 patients (based on endoscopic and histologic evaluation of the terminal ileum). Histological findings included villous atrophy, intra-epithelial lymphocytosis, and goblet cell deficiency which were identical to the duodenal pattern including the severity of the abnormalities. Finally, the colon was affected in two patients (case 3 and 11) in whom a mild-to-moderate colitis was observed.

Other autoimmune disease, auto-immune antibodies, and association with HLA- DQ 2.5

Six patients (46%) were previously diagnosed with an autoimmune disease: two patients with CD, two others with autoimmune thyroid disease, one with juvenile rheumatoid arthritis and one with type 1 diabetes (Table 1). Further underlining the susceptibility to autoimmune disease in these patients is the presence of one or more circulating auto antibodies, other than AEA, as found in 11 patients (85%). Most frequently found were parietal cell antibodies (5/13) including the two patients with AIG, antinuclear antibodies (4/13) and smooth muscle antibodies (3/13).

Two patients (case 9 and 10) had been diagnosed with CD 2 and 20 years before the onset of AIE diagnosis. Both patients had high titers of TGA (>100 U/ml) at the time of diagnosis. One patient was HLA-DQ2.5 heterozygous, and the other carried both the HLA-DQ2.5 and -DQ8 haplotypes. They initially responded to a GFD and at the time of reoccurrence of symptoms transglutaminase antibodies were undetectable. Histology showed villous atrophy with active chronic enteritis as well as apoptotic bodies in both. No findings in support of RCD type 1 or type 2 were found, i.e., no increased counts of γδ-IELs nor IELs with an aberrant phenotype.

Remarkably, nine of the 11 patients (82%) without a history of CD did carry one of the CD associated HLA genotypes: 7 were HLA-DQ2.5 heterozygous, and two were HLA-DQ8 heterozygous. TGA and EMA tested negative in all these patients at time of AIE diagnosis. Nevertheless, in order to exclude seronegative CD all 11 patients underwent a trial with a GFD but without clinical effect.

Treatment and response

Median follow-up of patients since AIE diagnosis was 56 months (range: 13 - 148 months). Four patients have died since AIE diagnosis. Three patients died due to severe malnutrition and cachexia as they failed to respond to various treatment strategies, whereas one patient died due to the complications of chronic myelomonocytic leukemia unrelated to AIE.

In order to maintain adequate nutritional status total parenteral nutrition was required in 8 cases (62%), while enteral tube feeding was sufficient in five other cases. For induction of remission various medications were used, and these included budesonide (n=5), prednisolone (n=8), ciclosporin (n=2), infliximab (n=1), rituximab (n=4) and cladribine (n=2). One therapy-refractory patient was eventually treated with autologous stem cell transplantation (auto-SCT) following high dose chemotherapy. Ambulant patients were treated with budesonide with alleviation of diarrhea in 60%. Patients with more severe disease that required hospitalization received prednisone therapy instead of budesonide with a clinical effect in 62%. Patients who failed on steroid therapy or were dependent on steroids due to recurrent disease received

various second line treatments. Rituximab, an anti-CD20 monoclonal antibody which directs against B-cells was administered in 4 patients (4 doses of 250mg) and was clinically effective in 2 patients.[19,20] Three other patients were treated with cladribine, a purine analogue, which has proven to be effective in another severe enteropathy, namely RCD type 2.[21] Two patients failed to show a clinical response to this treatment, but one patient (case 6) who was steroid-dependent (30mg prednisone per day) for four years as treatment with infliximab, rituximab and thiopurines failed, went into steroid free clinical remission lasting for over 3 years after one cycle of cladribine treatment. Ciclosporin and infliximab have shown promise in AIE but in three patients in our series failed to induce clinical effect.[5,22-24] One patient (case 1) that failed on prednisolone, cladribine, cyclosporine and tioguanin therapy and still suffered from severe malabsorption requiring total parenteral nutrition, underwent autSCT with a cyclofosfamide and anti-thymocyte globulin regimen, analogous with the treatment of RCD type 2.[25] This patient recovered clinically with normalization of stool frequency and weight gain, in addition to complete recovery of the duodenal histology. Currently, the patient is in remission for 140 months.

For maintenance therapy thiopurines were the mainstay of treatment. Patients were treated with tioguanin (n=6), mercaptopurin (n=2) or azathioprine (n=1). In six patients (67%) persistent remission, including normalization of stool frequency and consistency, >10% weight gain, and improvement of duodenal histology (according to the Mash criteria) was achieved with thiopurines. Two patients had to discontinue therapy at an early stage due to intolerance and acute pancreatitis, respectively. In one additional patient tioguanin had to be discontinued after 13 months due to increased serum liver enzymes. In three of the nine patients the drug failed to maintain a clinical, as well as a histologic effect. The two patients who did not tolerate thiopurines therefore received budesonide maintenance treatment which proved clinically effective.

Noteworthy, four patients (31%) are in long-lasting drug-free remission. Case 1 is still in clinical remissions 140 months after auto-SCT. Three others are in clinical remission without treatment for 28, 48 and 76 months respectively, after receiving immunosuppressive treatment for 48, 50 and 90 months.

DISCUSSION

Here we report on the experience of an European tertiary referral centre where 13 patients were diagnosed with AIE over a 13 year time-period. Due to the rarity and complexity of this disease diagnosis of AIE remains challenging as we show in this report.

Our case-series is the second largest after the series reported by the Mayo Clinic that initially described 15 adult onset AIE patients, and more recently reported on a total of 30 patients.[4,5] The authors report that AIE patients present with more severe diarrhoea and malabsorption-related symptoms than patients with RCD type 1.[4] Our series underscores the severity of AIE as almost two thirds of patients required total parenteral nutrition and three of the 13 (23%) died due to severe therapy refractory malabsorption.

One of the challenges in the differential diagnosis of AIE is the resemblance with coeliac disease. In our group of AIE patients the CD-related HLA-genotype HLA-DQ 2.5 was strongly overrepresented (79%). This is in contrast with the observation from the Mayo Clinic where the prevalence was similar to the general population (34% versus 35%).[4,5] Although considering the relatively small number of patients included in this study, the recently observed association of HLA-DQ2 with olmesartan-associated enteropathy draws attention to the role of HLA-DQ2 in immune-mediated enteropathies other than coeliac disease.[26] Not only are AEA found in 30% of patients with olmesartan-associated enteropathy but the histologic abnormalities observed in olmesartan-associated enteropathy can also mimic those in AIE.[27,28]

In line with previous reports AIE often occurred in patients with other autoimmune diseases, and circulating autoimmune antibodies were observed in the majority of patients.[5,29-31] These data support the suggestion that AIE is in fact a feature of a generalized hyperactive immune state.[32] Yet, the association with other immune-mediated enteropathies such as (refractory) coeliac disease is considered a clinical conundrum as differentiation among them is challenging.[5] Our experience strongly suggests that both enteropathies can indeed coexist as two patients previously diagnosed with CD later became nonresponsive to a strict GFD and developed AEA and histologic abnormalities corresponding with AIE but not to RCD. Furthermore, differentiating AIE from common variable immunodeficiency syndrome (CVID) can be challenging, but in our series no patients were diagnosed with CVID based on clinical presentation, serum immunoglobulin levels and duodenal histology.[5]

Histologic analysis of the duodenum showed classical features of AIE such as absence of goblet and Paneth cells and-or presence of increased numbers of apoptotic bodies in 11 out of 13 cases (86%). According to the histological classification of AIE the majority of patients (62%) were classified as 'active chronic enteritis' pattern (type 1), indeed the most common histologic pattern seen in AIE.[6] Underlining the heterogeneity in AIE is that the other cases corresponded with a CD-like (23%) or GVDH-like (15%). Notably, in 2 patients no villous atrophy was observed in the biopsies taken. Considering the severe malabsorption observed in these patients combined with other histologic features of AIE, high I-FAPB serum levels and their response to immunosuppressive treatment strongly suggest an immune-mediated enteropathy as the cause of the malabsorption. The most likely reason that villous atrophy was not seen

in their biopsies is sampling error due to patchy disease, or that deeper intubation into the small intestine is required.

The role of AEA in the pathogenesis of AIE is still unclear. In this study AEA titers did not correlate with severity of villous atrophy, confirming previous observations.[33] Yet, after initiation of treatment AEA did disappear in the majority of patients, even though a clear correlation with clinical or histologic response could not be established. Others have suggested that AEA are a secondary phenomenon that arise after initiation of T-cell mediated tissue damage of the intestine, as this may lead to the release of (auto)antigens which subsequently induce production of (auto)antibodies.[20] To address this we evaluated AEA in patients with other enteropathies, including CD, RCD type 2 and EATL. While absent in healthy controls, AEA were found in a low percentage of CD patients, and in up to 20% in EATL patients, thereby suggesting to be indeed secondary to (severe) enteropathy. This data is in contrast with a previous report that AEA are very specific in a population involving 2200 patients that underwent duodenal biopsy,[34] yet others have also found AEA in patients with OAE, inflammatory bowel disease and HIV infection.[15,35,36] In three patients the diagnosis of AIE was based on clinical and histologic features in absence of AEA as the latter are not absolutely required for the diagnosis.[5] One patient lacked both AEA and AGCA, while in two others AGCA were observed, findings in line with previous reports.[5,37,38] The relevance of this finding is however unclear, as AGCA can be found in up to 28% of patients with CD [39], and some experts suggest that AGCA should not be used for the diagnosis of AIE.[34]

Steroid therapy induced clinical remission in 62% of the patients in our study, which is similar to previous reports.[5] Depending on disease severity steroid treatment can vary from budesonide for mild symptoms to intravenous prednisolone for severe cases. When induction treatment with prednisone is successful, maintenance treatment with open capsule budesonide or thiopurines both seem reasonable options. In our series thiopurines were effective in 63% of patients when tolerated, and other series have shown promising effects for open capsule budesonide.[40] For patients refractory to steroid therapy it is currently unclear what the best step-up therapy is. This is illustrated in our study as these patients were treated with a variety of step-up therapy such as rituximab, ciclosporin, cladribine and infliximab. Due to small sample size no conclusions can be drawn from our and other series, especially since patients often received multiple treatments within a short timeframe making it impossible to identify the effective therapy. No single treatment was noted to be consistently effective in AIE patients. Therapies that have shown to be effective in our series are rituximab (2/4 patients) and cladribine (1/3 patients). Infliximab has been reported to induce clinical remission in four cases, although in our study it was ineffective in the one case it was tried.[5,22,24] Another promising therapy is vedoluzimab that regulates inflammation by blocking lymphocyte trafficking in the intestine and has been reported to be effective in adult-onset AIE.[41] Based on the currently available data we propose that patients refractory to prednisone should receive treatment with infliximab, rituximab or vedoluzimab (Figure 2). When severe malabsorption persists despite these various treatment strategies in patients aged under 70 years auto-SCT could be considered. We here describe the first patient with adult onset AIE that has been successfully treated with auto-SCT

currently being in a drug-free remission for over 10 years. In children with IPEX allogeneic stem cell transplantation (allo-SCT) is the only curative treatment available.[42] Immunopathogenesis in IPEX differs from AIE and is characterized by a mutated *FoxP3* gene resulting in dysfunctional regulatory $CD4^+$ $CD25^+$ $FOXP3^+$ T-cells and lack of control of autoreactive T- and B-cells in the affected patient.[43] A comparable gene mutation disturbing immunological regulation has not been found in AIE, however dysfunctional or reduced numbers of regulatory-cells may yet be involved in its pathogenesis. In general, allo-SCT is accompanied by a substantial higher (15-30%) transplant related morbidity and mortality than auto-SCT (<5%), which has directed treatment in severely ill patients with auto inflammatory disease towards the latter.[44] The rationale behind auto-SCT is that the conditioning regime followed by infusion of hematopoietic stem cells eradicates autoreactive immune cells allowing the generation of a de novo self-tolerant immune system.[45] Auto-SCT has proven safe and effective in another severe enteropathy, RCD type 2, and was therefore attempted in therapy-refractory AIE with severe diarrhea and wasting.[25,46]

When disease activity was under control maintenance therapy with thiopurines proved reasonable effective in our group. When thiopurines are not tolerated long term use of budesonide appears a good alternative.[40] Remarkably, in addition to the patient that was successfully treated with auto-SCT three other patients are in a long lasting drug-free clinical remission after being treated for 3-7 years with immunosuppressive therapy. Even though it is unclear what the natural course of the disease would have been, this data does suggests that immunosuppressive treatment can restore the equilibrium between pro-and anti-inflammatory responses in some AIE patients.

Development of EATL in patients with AIE has been sporadically reported, but this has not occurred in our case-series including 70,4 years of patient follow-up.[47,48] In contrast to RCD type 2 where phenotypical analysis can accurately identify a premalignant IEL population, in AIE no such population is present and patients are therefore screened with the less specific TCRG clonality analysis.[18] Caution is required when interpreting the relatively high number (38%) of monoclonal TCRG rearrangement patterns found in AIE patients, as clonal patterns were regularly found in patients with uncomplicated CD, possibly reflecting a dominant inflammatory T-cell clone rather than a malignant T-cell clone.[49,50]

Altogether, it is clear that adult-onset AIE occurs in genetic susceptible patients that are prone for autoimmunity, yet the group is remarkably heterogenic considering age at diagnosis, histological findings and the clinical course of disease. The considerable overlap of AIE with several other enteropathies with regard to histological findings, HLA-genotype and presence of AEA suggests, at least to some extent, a shared pathogenesis among those enteropathies. Furthermore, our data strongly suggests that there are multiple AIE associated auto-antigens, but it remains unclear whether they play a role in disease pathogenesis, and if so, whether different auto-antigens result in a different course of disease. Likewise, it is unknown wat triggers the inflammatory response in AIE, and whether this trigger (e.g. virus, or medications other than olmesartan and mycophenolate) is the same among patients. The long-lasting drug-free remission seen in a subgroup of AIE patients after long-term immunosuppressive

medication shows that the equilibrium in the immune response can be restored. Whether the fortuitous elimination of the so far unidentified trigger responsible for instigating this inappropriate immune response has contributed to the restored balance in these patients remains speculation at this point.

INDUCTION THERAPY

Step 1

Budesonide (open capsule)
Prednison when refractory to budesonide or in case of severe malabsorption

Step 2

Only sparse data that suggest a role for rituximab, anti-TNFα, cladribin and vedoluzimab

Step 3

For patients aged under 70 years with severe symptoms refractory to medication (step 1 and 2) autologous stem cell transplantation can be considered

MAINTENANCE THERAPY

Low dose budesonide or introduce a thiopurine

Figure 2 *Proposed treatment algorithm adult-onset autoimmune enteropathy.*

CONCLUSION

Adult-onset autoimmune enteropathy (AIE) is a rare but severe enteropathy that requires parenteral feeding in more than half of the patients and can lead to death due to severe therapy refractory malabsorption. While patients with AIE are prone to autoimmunity in general, serum anti-enterocyte antibodies might be secondary to the intestinal inflammation as they were found in patients with other enteropathies as well. This study further shows a high prevalence of HLA-DQ 2.5 in AIE patients. Remission induction treatment with steroids is effective in the majority of AIE patients and maintenance treatment consists of budesonide or thiopurines. In steroid refractory patients there is a variety of therapies that can be considered but none have been shown to be superior. In therapy refractory patients with severe symptoms aged under 70 years autologous stem cell treatment can be considered. After long-term immunosuppressive treatment one third of patients are in long-lasting drug-free clinical remission, which shows that adult-onset AIE is curable in a subset of patients.

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Chapter 4

Novel variant of EATL evolving from γδ T-cells in a RCD type 1 patient

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ABSTRACT

Objectives

Enteropathy-associated T-cell lymphoma (EATL) is a rare non-Hodgkin lymphoma that may complicate coeliac disease and typically occurs in patients with refractoriness to the gluten-free diet. The majority of these patients harbor a clonal expansion of intraepithelial lymphocytes (IEL) with an aberrant phenotype in the small intestine which are thus considered as the 'precursor' lymphoma cells. We describe a 51 year old female patient with refractory coeliac disease (RCD) who developed an EATL with manifestations in the proximal small intestine and in a mesenteric lymph node that did not evolve from regular type 'aberrant' αβ T-cells but rather from a clonal expansion of γδ T-cells.

Methods

Duodenal biopsies and lymphoma tissue from a patient with refractory celiac disease whom developed an EATL were extensively studied by immunophenotypical, T-cell receptor immunogenetic, and chromosomal analysis.

Results

Flow cytometric analysis of duodenal intraepithelial lymphocytes (IELs) revealed an unusual large clonal expansion of $CD30^-$ γδ T-cells in a RCD patient. When the patient clinically deteriorated 18 months later, a substantial part (30%) of this cell population did express CD30. In addition, identical immunogenetic aberrancies had developed in a pre-hepatic lymph node.

Conclusion

We here report on a case of extra-intestinal EATL that originated from a clonal γδ-IEL population rather than from aberrant IEL. This EATL displayed a distinctive pattern of immunophenotypical, T-cell receptor immunogenetic, and chromosomal aberrancies as compared to classical EATL, defining this lymphoma as a novel variant of EATL.

INTRODUCTION

Enteropathy-associated T-cell lymphoma (EATL) type I is a rare non-Hodgkin lymphoma that may complicate coeliac disease (CD). It is characterized by large pleomorphic and anaplastic cells that express the αβ T-cell receptor (TCR), CD30, CD4 and cytotoxic markers but not CD8 and CD56.[1] Type II EATL displays a distinctive morphology and immunophenotype and lacks a clear association with CD.[2] EATL can be diagnosed simultaneously with the diagnosis of CD or may develop in patients with a known history of CD. Typically, disease in the latter patients is non-responsive to the gluten-free diet (GFD) and is referred to as refractory coeliac disease (RCD).[3] Such patients display clonal expansion of a T-cell subset that occurs under physiological circumstances in low frequencies in the small bowel mucosa[4,5] which is characterized by the presence of cytoplasmic CD3 (cytCD3) but lacks surface expression of CD3, CD4 and CD8 (referred to as 'aberrant' T-cells cytCD3$^+$ CD3$^-$ CD45$^+$ CD7$^+$ CD4$^-$ CD8$^-$ cells).[6] In patients who develop EATL following a stage of RCD, a direct clonal relation of the EATL tumor cells to the clonally expanded 'aberrant' cells can be demonstrated based on identical TCR gene rearrangements.[7] Thus, such 'aberrant' cells can be considered precursor lymphoma cells.

Here, we report on a novel variant of EATL that did not evolve from regular type 'aberrant' T-cells but rather from an aberrant clonal duodenal γδ T-cell population.

Methods

Serum immunoglobulin (Ig)A levels, anti-tissue transglutaminase and anti-endomysial IgA and IgG antibodies were detected as previously described.[8] For HLA genotyping the alleles DQA1*05, DQA*02 and DQB1*02 (encoding the HLA-DQ2 heterodimers) and the alleles DQA1*03 and DQB1*0302 (encoding the HLA-DQ8 heterodimer) were determined by a polymorphism–heteroduplex assay, electrophoresis and gel-staining method on the PhastSystem (Amersham Pharmacia Biotech, Uppsala, Sweden) after polymerase chain reaction amplification.[5]

For histopathological evaluation biopsy specimens were fixed and preserved in 10% formalin. Histologic findings were classified using the Oberhuber's modification of the Marsh criteria.[5] Immunohistochemistry was performed on paraffin embedded tissue sections using standard procedures.

Flow-cytometric immunophenotypical analysis was performed on freshly isolated intraepithelial lymphocytes and peripheral blood lymphocytes as previously described.[5]

T-cell receptor (TCR) gamma chain and delta chain gene rearrangements were assessed by multiplex PCR and GeneScan analysis, as previously described.[5] For oligonucleotide array comparative genomic hybridization (Figure 3). Sample was hybridized to 180K Agilent microarrays (4x180k array, Agilent Technologies, Palo Alto, CA, USA, custom designed GEO platform GPL8687) accessible through http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GPL8687. Labelling and hybridization procedures were performed as previously described.[9] The experimental data has been made publicly available through GEO (GSE56425).[10]

The study was in accordance with the ethical guidelines of the VU University Medical Center and the patient gave written informed consent.

Case history (results)

A 51-year-old HLA-DQ 2.5 heterozygous female was referred to our center with severe diarrhea and weight loss. She had been diagnosed with CD 7 years earlier on the basis of anti-endomysium antibodies and subtotal villous atrophy (Marsh 3B). She had been on a GFD ever since and dietary adherence was confirmed by negative CD serology and evaluation by a specialized dietitian.

Duodenal histology showed Marsh 3B lesions with focal ulceration. Examination could exclude concomitant autoimmune enteropathy (AIE), immunodeficiency syndrome, Crohn's disease, parasitic infection and malignancy. Flow cytometric analysis of the duodenal IELs revealed a non-significant aberrant IEL population although an unusual large population of 76% γδ T-cells was present in the epithelial layer (compared to 1-35% in uncomplicated (R)CD patients).[8] These cells did not stain positive for CD30.

The patient was diagnosed with refractory CD without aberrant T-cells (RCD type 1) and started on prednisone and 6-tioguanine (6-TG). This resulted in normalization of the stool frequency, weight gain, and a follow-up biopsy six months later revealed almost complete mucosal recovery (Marsh 1); the percentage of γδ-IELs had decreased to 52%. Eighteen months later her clinical situation deteriorated and duodenal biopsies showed complete villous atrophy (Marsh 3C) and a persisting abnormally high γδ-IEL population (78%). Remarkably, FACS analysis now showed CD30 expression on 30% of the γδ-IELs while only 10% of αβ T-cells expressed CD30 within the normal range for activated T-lymphocytes. In the peripheral blood, no significant γδ T-cell population could be identified. PET-CT-scan showed diffuse increased FDG-uptake in the jejunum without further signs of lymphadenopathy or hepatosplenomegaly. In addition, a pre-hepatic intra-abdominal PET positive mass (diameter 15mmx9mm) was identified, which was not present on the CT scan of the abdomen that was performed 18 months earlier during the RCD workup.

Histological analysis

An excisional biopsy was taken from the lymph node. The lymph node showed a complete effaced architecture with a diffuse, sheet-like infiltrate of large lymphoid blasts with polymorphic nuclei, multiple large ovoid nucleoli and clear cytoplasm. Immunohistochemistry showed a very unusual immunophenotype (Figure 1). The tumor cells were uniformly positive for CD7, granzymeB, CD30 as well as CD20. TIA1 and CD15 were positive in a smaller percentage of the tumor cells. There was extensive T-cell marker loss as CD3, CD5, CD4 and CD8 were negative. Additional B-cell markers (CD79a and PAX5) were also negative. Epstein-Barr virus-encoded RNA (EBER) occasionally stained large cells, while the reactive small cell infiltrate showed more EBER positive cells . Gene-rearrangement studies showed a polyclonal immunoglobulin heavy chain rearrangement pattern but a monoclonal T-cell receptor gamma (TCRG) and delta (TCRD) rearrangements, confirming a T-cell origin of the proliferation. The monoclonal TCRG and TCRD rearrangement pattern in the tumor was identical to that observed in the duodenum (Figure 2), indicating that the monoclonal γδ T-cell population was already present in the duodenum at time of the initial RCD type 1 diagnosis .

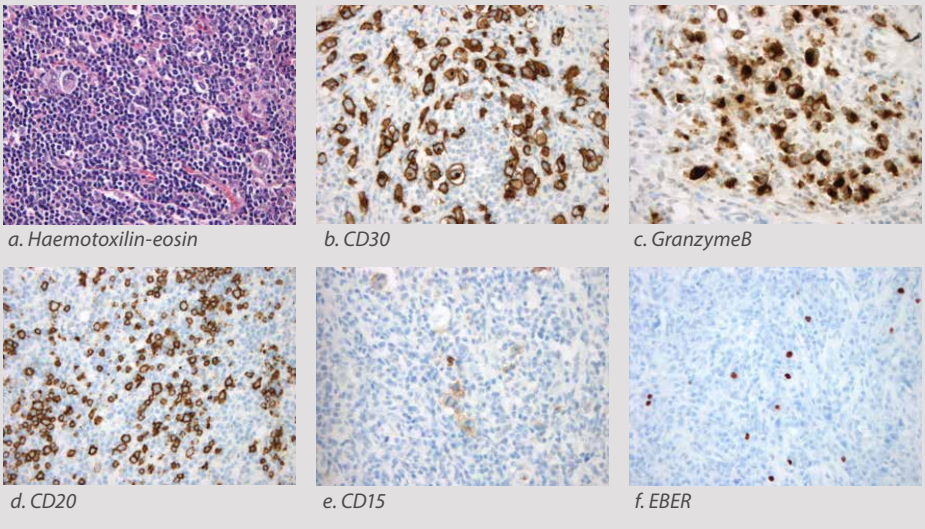

Figure 1 *Histology and immunophenotype of a novel variant of EATL.*

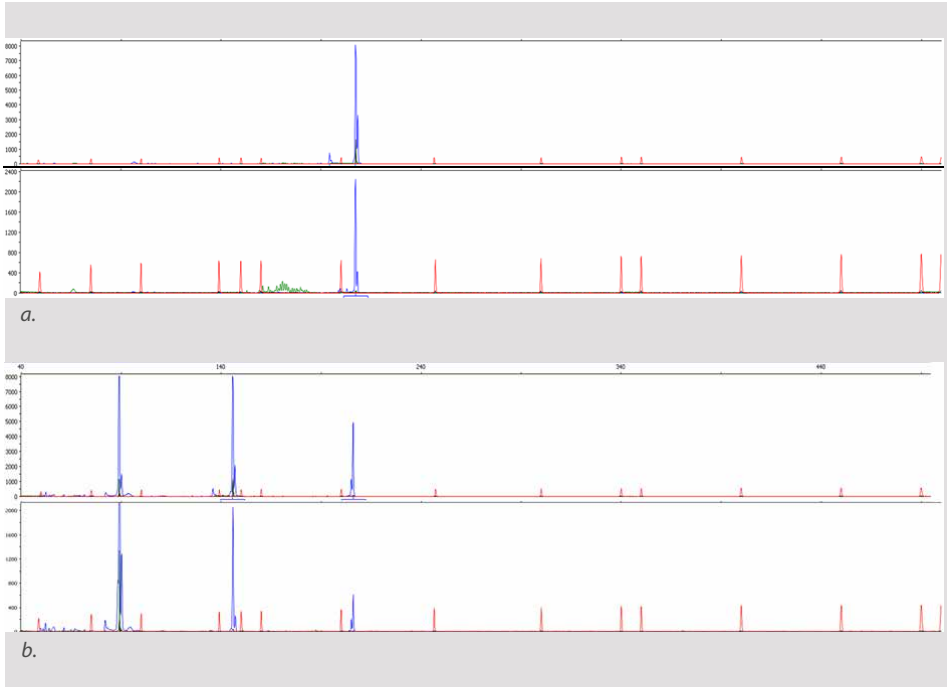

Figure 2 *Rearrangement analysis of the TCRG **(a)** and TCRD **(b)** genes on DNA derived from a duodenal sample 2 years prior to tumor development (top) and the extra-intestinal EATL (bottom).*

Genetic studies

Array comparative genomic hybridization (aCGH) revealed only one chromosomal gain on the chromosome region 7q36 (Figure 3). Fluorescent in situ hybridization for MYC did not show a MYC break .

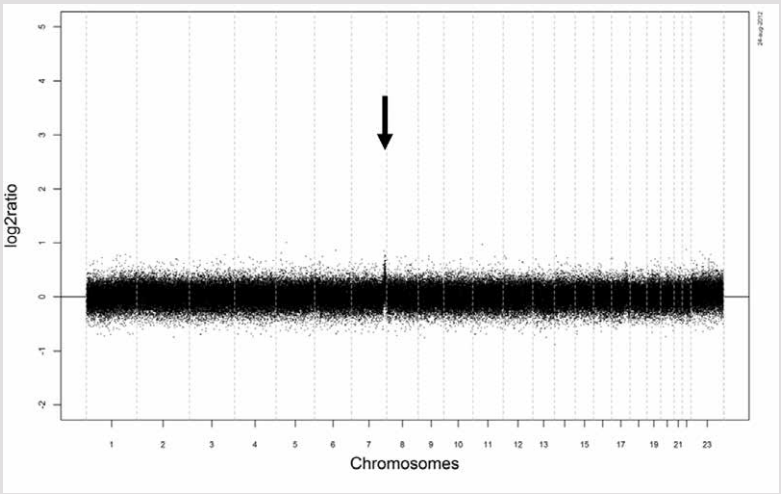

Figure 3 *Chromosomal imbalance in the enteropathy-associated T-cell lymphoma as detected by array comparative genome hybridization. Only at 7q36 a chromosomal aberration is visible. The X-axis represents the genomic locations of the probes on the array in Mb and the Y-axis represents the log2 ratios of the probes.*

Treatment

Initially, the patient was treated with a five-day course of Cladribine 0.1mg/kg/day. Malabsorption related symptoms did not improve and villous atrophy persisted. Therefore, the patient was subsequently treated with 3 courses of rituximab, cyclophosphamide, hydroxydaunorubicin, oncovin en prednisone (R-CHOP). This resulted in clinical improvement as defecation frequency normalized and a four kilograms weight gain was reached after three months. A follow-up PET-scan revealed a diminished lymphoma lesion. The duodenal mucosa showed complete recovery (Marsh 0). Moreover, the γδ T-cell population decreased to 29% of total IELs, a percentage that is considered normal in (R)CD patients, with absence of CD30 expression.

Currently, 2 years after R-CHOP treatment, the patient is clinically well and repeated follow-up endoscopy showed normal mucosa without histological abnormalities. The γδ-IEL population varies between 25 and 30% of total IELs.

DISCUSSION

This case involves a CD patient who developed a T-cell lymphoma with an unusual phenotype and genotype. As far as we are aware, this is the first report of an extra-intestinal lymphoma originating from a duodenal γδ-EL population. Peripheral γδ T-cell lymphomas frequently concern hepatosplenic T-cell lymphomas but our patient had dissimilar clinical, phenotypical and chromosomal characteristics. Intestinal γδ T-cell lymphomas have been reported but consist of monomorphic cells with a $CD103^+$ $CD3^+$ $CD8^+$ $CD56^+$ phenotype that can be categorized as type II EATL and are not associated with CD.[11]

The observed genetic aberrations in this patient involved gain of the 7q region, which shows frequent alteration in EATL as well as in many other T- and B-cell lymphomas.[12,13] Other common chromosomal aberrations observed in EATL type I and II, including gains in 1q, 5q and 9q, were absent in this patient (Table 1).

Table 1 *Overview of the characteristics of the at presently reported various types of EATL.*

Characteristics	EATL type I	EATL type II	variant type EATL	AIE-associated T-cell lymphoma
Association with enteropathy	+	-	+	+
HLA-DQ2/-DQ8	+	-	+	+
Putative precursor cell	$sCD3^-$ IEL	clonal aberrant T-cell	clonal γδ T-cells	clonal CD8 αβ T-cells
Morphology	polymorphic	monomorphic	polymorphic	monomorphic
Phenotype:				
-CD3	+	+	-	+
-CD4	mostly -	-	-	-
-CD5	mostly -	mostly + (70%)	-	+
-CD8	mostly - (20%+)	+	-	+
-CD7	+	+	+	-
-CD15	-	-	+	nd
-CD20	-	-	-	-
-CD30	mostly + (80%)	-	+	+/-
-CD56	-	+	-	-
-Granzyme	+	+	+	+
-TIA1	+	+	+	+
Chromosomal aberrantions:				
-gains	1q, 5q, 7q, 9q,	MYC, 7q, 8q, 9q	7q	7q
-loss	8p, 13q,16q			8p, 10q

Moreover, aberration of the MYC oncogene, as often present in type II EATL, was absent. In addition, the recently described EATL that arose in an AIE patient who also displayed aberrations on chromosome 7 involved a distinctly more complex pattern with alterations on 8p22-23.2 and 10q23 which, again, were absent in our patient.[14] The 7q region consists of a highly dense gene region that includes multiple genes with regulatory functions and it is tempting to speculate that these play a role in the onset of this tumor.

The current classification for EATL is unsatisfactory as it is becoming clearer that EATL type II is not associated with enteropathy and seems a separate entity rather than a variant of type I EATL.[2] In addition, as in the case described here, the AIE-associated EATL as well as other intestinal lymphomas with a broader spectrum of this disease[15] clearly show a need for a more elaborate division (Table 1). The precursor cells from which these various EATLs arise form an important area of research to better understand these lymphomas.

CONCLUSION

In conclusion, we here report a novel variant of EATL, with an unusual phenotype and genotype in a RCD type 1 patient, that evolved from a clonal γδ-IEL population. This case provides additional support for the much needed development of a novel classification for EATL.

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PART II

DIAGNOSTIC ASPECTS

Chapter 5a

Accurate classification of RCD requires flow cytometry

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Letter to the editor. Gut 2010 Dec;59(12):1732.

We read with interest the article of Liu et al in Gut, in which the authors emphasize the need for monitoring of clonality and intraepithelial lymphocyte (IEL) immunophenotype in the surveillance of refractory coeliac disease (RCD).[1]

The authors state there is no consensus on the cut-off of aberrant cells distinguishing between non-complicated coeliac disease (CD), RCD type 1 and RCD type 2. However, in 2000 it was shown that based on the number of aberrant T-cells, CD can be distinguished from RCD by immunohistochemistry.[2] Furthermore, using flow cytometry, Verbeek defined a clinically well-validated cut-off of 20% IELs as being diagnostic for RCD type 2.[3]

In their paper, the authors describe that a high percentage of patients (80%) progress from RCD type 1 to type 2. This is somewhat surprising since studies so far have indicated that transition from RCD type 1 to RCD type 2 or EATL (enteropathy-associated T-cell lymphoma) is a rare phenomenon. In fact, in our institute >100 patients with RCD have been analysed using flow cytometry with a follow-up of 10 years. So far, only one patient with RCD type 1 transformed to RCD type 2 (C J Mulder, personal communication in 2010). This rare transition is reflected by the favourable 5-year survival of patients with RCD type 1 compared with those with RCD type 2.[4] If the occurrence of this transformation were as common as suggested, the 5-year survival would be expected to be significantly lower.

A potential explanation for these contradictory results may relate to the fact that the methodology used in this study has well-known limitations and potential pitfalls in the identification of aberrant T-cells in the gut. The most important problem is that immunohistochemistry does not allow differentiation between surface CD3 and cytoplasmatic CD3, and consequently the identification of aberrant IELs is solely based on the absence of CD8 in CD3-positive cells. However, $CD3^+$ $CD4^+$ T-cells comprise a considerable percentage of the IEL population, both in normal duodenum and in patients with CD and RCD.[3] According to the criteria of Liu et al, these cells would have been classified as aberrant T-cells. Furthermore, γδ-lymphocytes express a similar $CD3^+$ $CD8^+$ marker pattern to aberrant IELs and therefore these cells may also be erroneously classified as aberrant IELs. This is particularly relevant since γδ-cells comprise up to 50% of the IEL compartment, with increased percentages in active CD, and drastically decreased numbers in RCD type 2.[3] Finally, we sometimes encounter patients with aberrant ($sCD3^-$ $cytCD3^+$) T-cells that do express sCD8, which would have been classified as normal cells using immunohistochemistry. Including $CD3^+$ $CD4^+$ T-cell and γδ-lymphocyte populations in the enumeration of the aberrant T-cell population using immunohistochemistry leads to a relatively high cut-off value as compared with flow cytometry. Consequently, it cannot be excluded that in the study of Liu et al a substantial number of patients already had increased baseline numbers of aberrant T-cells that did not exceed the cut-off value of 40% and thus were initially misclassified as RCD type 1. In a later phase, when disease develops and the percentage of aberrant cells increases further, patients are diagnosed as RCD type 2. This would explain the relatively high number of transitions from RCD type 1 to RCD type 2.

Whereas patients with RCD type 1 have an excellent prognosis, up to 60% of patients with RCD type 2 will progress to EATL with a very poor outcome. Cladribine treatment and/or autologous stem cell transplantation may delay or even prevent the development of EATL. A correct initial investigation is therefore of utmost importance. So far there are no head-to-head comparisons between immunohistochemistry and flow cytometric evaluation of aberrant T-cells. Based on the aforementioned considerations we feel that flow cytometry is a validated, easy applicable methodology for the enumeration of aberrant T-cells that is superior to combined T-cell receptor gene clonality analysis and immunohistochemistry.

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Chapter 5b

Optimal strategies to identify aberrant intraepithelial lymphocytes in refractory coeliac disease

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J Clin Immunol. 2014 Oct;34(7):828-35.

ABSTRACT

Introduction

Different strategies have been developed to identify those refractory celiac disease (RCD) patients who are at risk to develop an enteropathy-associated T-cell lymphoma (EATL). Flow cytometric analysis of intraepithelial lymphocytes (IEL) with an aberrant phenotype is considered the golden standard yet is not widely available. Immunohistochemistry (IHC) and T-cell receptor (TCR) gene rearrangement studies are commonly available but may lack sensitivity and specificity. Here, we compared the three different methods in the workup of patients suspected for RCD.

Methods

Duodenal biopsies from control patient (n=5), RCD patients with moderately increased aberrant IEL populations (20-50%: n=14), and RCD patients with high numbers of aberrant IEL (>50%: n=5) as determined by flow cytometry, were analysed by IHC and TCR gamma chain rearrangement analysis. Three pathologists scored the slides independently.

Results

Concordance between pathologists in the IHC score was 74%. Sensitivity of IHC and TCR gamma rearrangement analysis in RCD patients with high numbers of aberrant IELs was 100% and 71%, respectively. RCD patients with aberrant cells between 25 and 50% however, were missed by IHC and TCR in 50% and 57% of cases, respectively.

Conclusion

Immunohistochemistry and to a lesser extent TCR gamma clonality analysis are sensitive in identifying patients with high numbers of aberrant IEL populations, yet miss half of RCD patients with moderately increased numbers. In addition, IHC has a high inter-observer variability. Therefore, patients suspected for RCD should undergo flow cytometric analysis of the duodenum.

INTRODUCTION

Coeliac disease (CD) patients with persisting or recurring symptoms despite strict adherence to a gluten-free diet (GFD) and absence of other concomitant intestinal diseases are diagnosed with refractory coeliac disease (RCD).[1] It affects less than 1% of CD patients yet results in increased morbidity and mortality.[2] While already in 1962 a link between CD and enteropathy-associated T-cell lymphoma (EATL) was reported,[3] it only recently became apparent that RCD patients in particular have a high chance to develop an EATL.[4] This was further substantiated by the observation that EATL is often preceded by the appearance of a clonal expansion of an intraepithelial lymphocyte (IEL) population in the duodenal mucosa that displays an abnormal or 'aberrant'phenotype.[5] These aberrant IELs lack normal cell surface expression of CD3 or CD8, yet have the cytoplasmatic CD3 (cytCD3) and T-cell receptor (TCR) molecules contained inside the cell. Such cells are present in small numbers under physiological circumstances and appear to represent a unique cell subset.[6] Clonal expansion of this cell population has only been found in a subgroup of RCD patients and EATL patients.

RCD patients can be further classified according to the number of aberrant IELs. This is clinically relevant since patients with low numbers of aberrant IELs, defined as less than 20% of total IEL upon flow cytometry, are not at increased risk to develop EATL and have a normal 5-year survival.[7,8] These patients are referred to as RCD type 1. When these numbers exceed this threshold (referred to as RCD type 2) the risk to develop EATL is dramatically increased with a poor 5-year survival of 44-58%.[7,9-13] That these cells are precursors of lymphoma cells was demonstrated by the observation that the TCR rearrangement repertoire is similar in sequential biopsies from RCD type 2 patients who developed an EATL.[9] So far, no correlation has been found in this patient category between the percentage of aberrant IELs and lymphoma risk.[14] Since timely diagnosis and treatment have shown to reduce the development of EATL in RCD type 2 patients it is of utmost importance to adequately diagnose and classify these patients.[1]

Currently, three methods are available to identify aberrant IELs: immunophenotypical analysis by either immunohistochemistry or flow cytometry, and clonality analysis by TCR rearrangement studies.[15] Flow cytometric analysis is considered the golden standard due to its ability to distinguish different IEL subsets.[8] Nevertheless, this technique is available in only a few specialized centres. Clonality analysis of the TCR gamma chain (TCRG) and immunohistochemistry (IHC) are commonly available and easy applicable techniques to identify aberrant IEL populations yet the accuracy of these techniques to diagnose RCD is unknown.[16]

Here we compare the ability of these three diagnostic modalities to accurately determine abnormal IEL populations.

METHODS

Patient selection

Patients visiting the gastroenterology department at the VU University Medical Center between January 2006 and December 2012 were included in this study. Patient characteristics are described in Table 1.

Table 1 *Patient characteristics.*

Group	Total number of patients	Gender F / M	Age (years) median; range	TGA and-or EMA POS / NEG
Controls	5	3 / 2	37; 24-46	0 / 5
RCD II moderate	14	6 / 8	59; 31-71	0 / 14
RCD II high	5	4 / 1	67,5; 57-76	0 / 5
$CD4^+$IEL population	1	0 / 1	42	0 / 1
γδ-IEL population	1	0 / 1	75	0 / 1

Abbreviations: **TGA** anti-tissue transglutaminase antibodies; **EMA** anti-endomysium antibodies.

1) Control patients without CD (patients 1-5). These patients have been described elsewhere,[8] and underwent upper gastrointestinal endoscopy for exclusion of CD due to symptoms that varied from aphtous stomatitis, gastric reflux disease, nausea and dyspepsia to diarrhoea, abdominal pain and osteopenia. All patients lacked circulating anti-endomysium (EMA) and/or anti-tissue-transglutaminase antibodies (TGA) and (histological) duodenal abnormalities. Five patients were randomly selected. 2) RCD type 2 patients with a moderate aberrant IEL population (between 20% and <50%: patients 6-19). These patients have been described elsewhere.[14] Diagnosis of RCD type 2 was based on presence of HLA-DQ2.5 and/or DQ8, initial serum EMA and-or TGA or initial (clinical) response upon GFD, and disappearance of CD-related antibodies upon GFD with persistence or recurrence of symptoms and CD-associated histological abnormalities in absence of concomitant small intestinal disease. 3) RCD type 2 patients with a high percentage aberrant IELs (>50%: patients 20-24). Similar to group 2. Biopsy specimens from RCD type 2 patients in group 2 and 3 included patients in follow-up who had received treatment with cladribin and possibly autologous stem cell transplantation and in response showed histological recovery, while mucosal invasion with aberrant IELs persisted (case 8-14, 16, 18, 19, 22). 4) Patients with abnormal high percentages of intraepithelial $CD3^+$ γδ T-cells or $CD3^+CD4^+$ T-cells (patients 25,26). Case 25 was diagnosed with a non-CD, immune-mediated enteropathy with a high percentage of $CD3^+$ $CD4^+$ IELs.[17] Case 26 was diagnosed with CD based on architectural abnormalities (Marsh 3B) and presence of TGA and received control endoscopy after four years of GFD due to recurrence of symptoms. Duodenal biopsy specimens showed high numbers (53%) of $CD3^+$ γδ T-cells in the epithelium.

Sample selection

During upper gastrointestinal endoscopy multiple large spike forceps biopsies were taken from the second part of the duodenum. Four biopsy specimens were fixed in 10% formalin for histological evaluation and immunohistochemistry and six biopsies were used for immediate flow cytometric evaluation.

Flow cytometric analysis

IELs were isolated from the biopsies as previously described.[14] Briefly, biopsies were vigorously shaken at 37°C for 60 min in PBS supplemented with 1 mM DTT (Fluka Bio- Chemika, Buchs Switzerland) and 1 mM EDTA (Merck, Darmstadt Germany). The released IELs were washed twice with PBS supplemented with 0.1% BSA (Roche Diagnostics) and stained for 30 min on ice, with fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridinin chlorophyll protein (PerCP) and allo-phycocyanin (APC)-labeled monoclonal antibodies directed against CD3, CD4, CD7, CD8, CD16 + 56, CD19, CD45, (all from BD Biosciences, San Jose, CA), Cytoplasmic staining of CD3 was performed after cell permeabilisation by Cytofix/CytoPerm Plus (BD Biosciences). A standard 4-color flowcytometer was used for analysis (FACS Calibur, BD Biosciences). The clinically validated cut-off value of >20% 'aberrant' IELs ($cytCD3^+CD3^-CD4^-CD8^-CD16^-CD56^-CD45^+$) as percentage of total intestinal lymphocytes was considered abnormal.[8] With the purpose to gain further insight in the test characteristics and patients of interest, an arbitrary differentiation was made regarding the size of the aberrant IEL population: a 'moderate' aberrant IEL population (20% < aberrant IEL of total $CD45^+$ IEL < 50%) and a 'high' aberrant IEL population (>50% aberrant IEL of total $CD45^+$ IEL).

Immunohistochemistry

Two subsequent slides were selected. After deparaffinisation with xylene and alcohol, endogenous peroxidase was blocked using 3% H2O2-methanol solution. Retrieval of antigens was carried out using high pressure-cooking in TRIS/EDTA buffer (pH=9.0 for 10 minutes), followed by double staining with mouse monoclonal antihuman CD8 antibody using an avidin–biotin peroxidase / 3,3'-Diaminobenzidine (DAB) kit (Sigma, St. Louis, USA) whilst CD3 was identified with a rabbit polyclonal antihuman CD3 antibody (DAKO, Heverlee, Belgium) using the alkaline phosphatase/fast blue reaction for 45 minutes (Sigma, St. Louis, USA). Cells were hereafter counterstained with haematoxylin for 10 seconds.

Determination of aberrant IEL

Two gastrointestinal pathologists and one pathology resident blindly scored the slides. These pathologists were provided with slides containing two duodenal samples and were instructed to randomly score an area covering a minimum of 100 enterocytes. Subsequently, the total number of IEL, CD3, and CD8 positive IELs were scored. The test was considered normal when more than 50% of all $CD3^+$ IELs co-expressed CD8 (<50% $CD3^+CD8^-$), and abnormal when less than 50% of all CD3+ IELs co-expressed CD8 (>50% $CD3^+CD8^-$).[18]

T-cell receptor gamma (TCRG) gene rearrangements

Analysis for the presence of TCRG gene rearrangements was performed on DNA isolated from cryopreserved biopsy specime ns using IdentiClone™ TCRG Gene Clonality Assay (Invivoscribe Technologies, Inc, La Ciotat, France) according to the recommendations of the manufacturer. In three patients (case 3,4 and 5) in the control group no cryopresevered specimens were available.

Ethical approval

All biopsy specimens were obtained for diagnostic purposes and the procedures were in accordance with the ethical guidelines of our institution.

Statistical analysis

For each group the median percentage and the range of the percentages of aberrant IELs and $CD3^+$ $CD8^-$ IELs were calculated for the flow cytometric and the immunohistological analysis, respectively. Furthermore, the sensitivity and specificity of IHC and clonality analysis were calculated and compared to the flow cytometric analysis.

RESULTS

Aberrant IELs are characterized by cytoplasmatic expression of CD3 and absence of CD3 and CD8 on the cell surface. Immunohistochemistry can visualize CD3 and CD8 expression without being able to differentiate between cytoplasmic and cell surface expression. This methodology can therefore not distinguish between aberrant (cyt$CD3^+$ $CD3^-$ $CD8^-$ cells) and 'normal' cell surface CD3 positive cells that also lack CD8 expression. The latter include $CD3^+$ γδ T-cells and $CD3^+$ $CD4^+$ T-cells. The number of $CD3^+$ γδ T-cells are particularly elevated in the epithelium of CD patients, and can comprise up to 50% of cells in this compartment. To avoid a false positive diagnosis, RCD type 2 is therefore arbitrarily defined as >50% $CD8^-$ cells of all $CD3^+$ IEL when IHC is applied. Flow cytometry is able to differentiate between cytoplasmic and surface expression of CD3. Previous work has shown that the population of cytoplasmic $CD3^+$ cells without surface CD3 never exceeds 20% in CD patients whereas an aberrant IEL population higher than 20% is only observed in RCD type 2 patients and associated with EATL development (illustrated in Figure 1).[8]

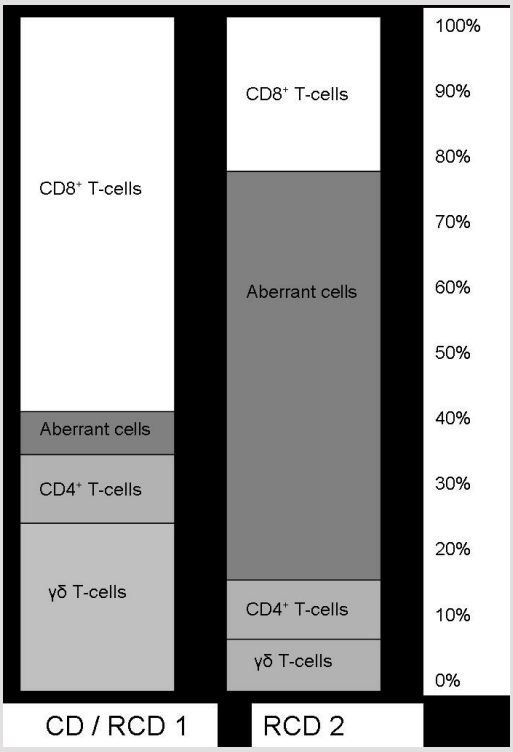


Figure 1 *Distribution of intraepithelial lymphocyte (IEL) populations with $CD3^+$ $CD8^-$ phenotype, typical for patients with coeliac disease and refractory coeliac disease type 2. The percentage of $CD3^+$ γδ T-cells, $CD3^+CD4^+$ T-cells and aberrant IELs of the total IEL populations is depicted.*

Control patients

As expected, flow cytometric analysis showed small amounts of aberrant IELs in the control population (median of 8%: range: 7-12%).[5,8] Using immunohistochemistry, a much higher variability was found in this group, ranging from 11 to 73% $CD3^+CD8^-$ IELs (Table 2). Slides from three individuals were unanimously scored as normal, while two slides were scored as having increased numbers of aberrant IELs by one out of the three pathologists .

Immunohistochemistry misses half of the RCD type 2 patients with a moderate aberrant IEL population

In the group of RCD type 2 patients, the number of aberrant IELs varied between 20 and 92% as determined by flow cytometry. The slides from all five patients with high percentages of aberrant IEL populations above 50% upon flow cytometry (median 81%; range 60-92%), were unanimously scored as RCD type 2 upon immunohistochemistry (sensitivity of 100%: Table 2

In the group of RCD type 2 patients with a moderate aberrant IEL population (median 34: range 20-50%) there was much higher variability in the IHC analysis. Seven out of 14 patients were classified as having normal amounts of $CD3^+$ $CD8^-$ IELs by at least two pathologists. Thus, 50% of patients with moderately increased numbers of aberrant IELs may be missed in the IHC analysis.

Immunohistochemistry is reasonably specific yet misses atypical cells

To determine the specificity of immunohistochemistry two cases without aberrant IELs, but with inflammatory changes in the intraepithelial compartment were evaluated. These included a patient with a non-CD, immune-mediated enteropathy with a $CD3^+$ $CD4^+$ IEL infiltrate (case 25) and a CD-patient with a dominant $CD3^+$ γδ T-cell infiltrate (case 26). These patients did not reveal increased aberrant IELs upon flow cytometric analyses yet were unanimously scored as having increased numbers of $CD3^+$ $CD8^-$ T-cells upon immunohistochemistry by both pathologists (Table 2).

Inter-observer variability

In 10/26 (38%) of all slides evaluated in this study there was a discordance in the final test result. This was especially apparent in the group of RCD type 2 patients with moderate aberrant IEL population. In 8 out of 14 patients (57%) the diagnosis varied between the pathologists.

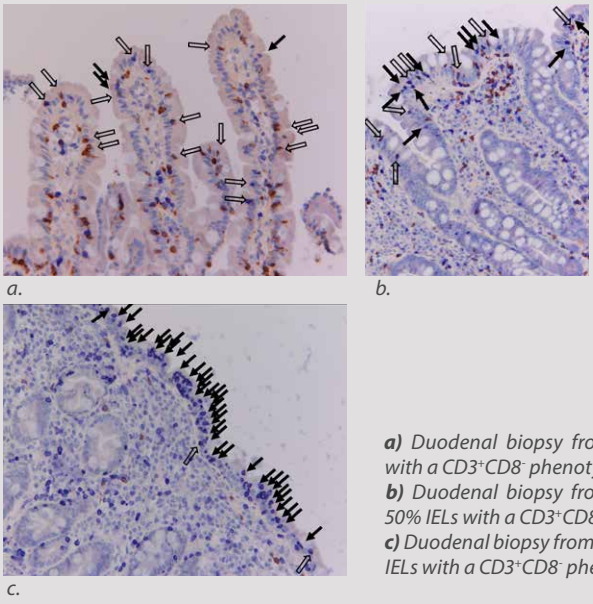
TCRG gene rearrangement studies

Overall, 12 out of 22 individuals tested displayed a monoclonal TCRG rearrangement. Quite unexpectedly, these included two control samples. Due to insufficient material clonality analysis was not possibly in the other three control patients. In the RCD type 2 group with moderate numbers of aberrant cells, 6 out of 14 (sensitivity 43%) patients displayed a monoclonal pattern. In the group with high percentages of aberrant IEL 4 out of 5 patients had a monoclonal pattern (table 2). In this subgroup the sensitivity of TCRG clonality analysis was 80%.

Table 2 *Overview of test scores.*

Case	Group	Pre-treatment	Flow cytometry	Marsh score	Immunohistochemistry (% $CD3^+CD8^-$ IEL)				TCRG analysis
			% ab-IEL		P1	P2	P3	Test score A / N	Clonality
1	Control patient	-	7%	0	40%	48%	13%	0/3	M
2	Control patient	-	8%	0	21%	53%	30%	1/2	M
3	Control patient	-	8%	0	37%	47%	11%	0/3	ND
4	Control patient	-	11%	0	36%	40%	12%	0/3	ND
5	Control patient	-	12%	0	29%	73%	35%	1/2	ND
6	RCD 2 moderate	-	20%	3B	61%	74%	46%	2/1	P
7	RCD 2 moderate	-	20%	3A	88%	85%	53%	3/0	P
8	RCD 2 moderate	2-CDA	21%	2	42%	59%	15%	1/2	P
9	RCD 2 moderate	2-CDA	23%	2	40%	67%	14%	1/2	P
10	RCD 2 moderate	2-CDA	25%	0	27%	45%	0%	0/3	P
11	RCD 2 moderate	2-CDA + ASCT	27%	2	23%	62%	10%	1/2	M
12	RCD 2 moderate	2-CDA	33%	0	25%	68%	76%	2/1	P
13	RCD 2 moderate	2-CDA + ASCT	34%	0	93%	89%	91%	3/0	M
14	RCD 2 moderate	2-CDA	42%	0	41%	74%	28%	1/2	P
15	RCD 2 moderate	-	45%	3C	87%	81%	85%	3/0	M
16	RCD 2 moderate	2-CDA	45%	2	62%	67%	56%	3/0	M
17	RCD 2 moderate	-	47%	3C	94%	85%	92%	3/0	P
18	RCD 2 moderate	2-CDA	48%	0	46%	68%	29%	1/2	M
19	RCD 2 moderate	2-CDA	50%	1	47%	72%	21%	1/2	M
20	RCD 2 high	-	60%	3A	81%	-	91%	2/0	M
21	RCD 2 high	-	69%	3A	88%	83%	80%	3/0	P
22	RCD 2 high	2-CDA	81%	1	96%	95%	66%	3/0	M
23	RCD 2 high	2-CDA	84%	3A	97%	98%	63%	3/0	M

*Scoring of duodenal slides of controls, patients with refractory coeliac disease type 2 (RCD 2) and patients with large $CD3^+CD4^+$ or $CD3^+$ γδ-IEL populations. The * symbol indicates that the duodenal sample derived from a RCD type 2 patient in follow-up. Depicted is the percentage of aberrant IELs (ab-IEL) of the total $CD45^+$ IELs as determined by flow cytometry, the Marsh score of each slide containing a duodenal biopsy that was scored by pathologist (P) 1,2 and 3. The percentage of $CD3^+$ CD8- IELs of the total $CD3^+$ IELs is presented per pathologist. The 'test-score' shows how many pathologists scored the slide as abnormal (>50% $CD3^+CD8^-$ IELs of total $CD3^+$IELs) or normal (<50% $CD3^+CD8^-$ IELs of total $CD3^+$IELs). Results from TCRG gene rearrangement analysis are presented as monoclonal (M), polyclonal (P) or not determined (ND).*



a.

b.

c.

a) *Duodenal biopsy from healthy control: 8% IELs with a $CD3^+CD8^-$ phenotype*
b) *Duodenal biopsy from RCD type 2 patient with 50% IELs with a $CD3^+CD8^-$ phenotype*
c) *Duodenal biopsy from RCD type 2 patient with 90% IELs with a $CD3^+CD8^-$ phenotype*

Figure 2 *Slides from duodenal biopsies were primarily stained for CD8 using DAB (brown), and subsequently for CD3 using the alkaline phosphatase reaction (blue).*

DISCUSSION

RCD is a rare disease entity in which the differentiation between the benign type 1 and the pre-malignant type 2 is crucial. A generally available method to diagnose RCD type 2 is CD3/CD8 staining by immunohistochemistry.[18] Our data confirm the ability of this method to reveal dominant aberrant IEL populations that make up over 50% of IELs.[11,18] However, in patients with a moderate aberrant IEL population (between 20% and 50%), a significant number of patients will be missed and thus may deprive RCD type 2 patients from timely treatment.[16,19] In this regard it is important to realize that once the threshold is surpassed there is no correlation between the percentage of aberrant IELs and the risk of EATL development.[14,20] The lack of sensitivity of immunohistochemistry can be largely explained by the inability of IHC to distinguish between cell surface and intracellular expression of CD3, the latter being the hallmark of aberrant IELs. This is relevant since a $CD3^+ CD8^-$ phenotype includes $CD3^+ CD4^+$ T-cells as well as $CD3^+$ γδ T-cells. To avoid false-positives the threshold for the classification of RCD type 2 is therefore defined as >50% $CD3^+CD8^-$ of all $CD3^+$ IELs,[18] whereas a cut-off value of only 20% is used with flow cytometry.[8] A further complicating factor in the interpretation of IHC results comes from the observation that there is an inverse correlation between the percentage of $CD3^+$ γδ T-cells and the percentage of aberrant IEL in RCD type 2 patients (Figure 1).[21] Since both cell populations are scored as aberrant due to their $CD3^+ CD8^-$ phenotype by IHC, an increase of aberrant IEL can be compensated by a concomitant decrease of benign $CD3^+$ γδ T-cells, which can prevent the total amount of aberrant IELs to exceed the cut-off level.

In addition to a lower sensitivity, IHC may lack specificity. This was illustrated by a CD patient with a large benign population of $CD3^+$ γδ-IEL and a patient with non-responsive CD with an influx of $CD3^+ CD4^+$ IEL due to another underlying disease who were both erroneously classified as RCD type 2. Although we and others[22] have not been able to perform reliable staining procedures for these markers on paraffin fixed tissue specimens, there is a recent report where immunohistological staining of γδ T-cells in paraffin embedded duodenal biopsies has been successful.[23] This development may lead to a better test performance of IHC in the characterization of aberrant IELs.

One surprising finding of this study is the large inter-observer variability of IHC. A potential explanation relates to the fact that there is a certain degree of variation in cellular infiltrate throughout the specimen. Numbers produced by flow cytometric analysis represent an average of six biopsy specimens.

The lower sensitivity of immunohistochemistry must be outweighed against the fact that this technique is easy applicable and generally available, whereas flow cytometric analysis of IEL requires fresh duodenal biopsies and skilled analysts. Nevertheless, flow cytometry is nowadays available in most hospital laboratories and the cost of this analysis is not disproportionately higher when compared to IHC.

The sensitivity of TCRG rearrangement analysis to identify RCD patients that later developed an EATL has been reported as 67-78%.[8,24] We[20] and others showed that additional analysis of the TCR beta chain[25] or the TCR delta chain[11] may improve sensitivity. Nevertheless, even combined

clonality analysis of the gamma, beta and delta chain fails to identify all clinically relevant IEL populations with an aberrant phenotype.[20] Our data suggest that the likelihood of identifying a monoclonal expansion correlates with the percentage of aberrant IELs. None of the patients with 20-25% aberrant T-cells were found to have a clonal IEL population. Whether this is due to a lack of sensitivity of TCR clonality analysis to recognize this clone within the abundant T-cell infiltrate, or whether the enlarged aberrant IEL population initially expands poly- or oligoclonal is unclear.

CONCLUSION

In conclusion, the commonly used CD3/CD8 ratio as determined by immunohistochemistry and to a lesser extent TCRG clonality analysis are reliable tools to identify dominant aberrant IEL populations, but fail to reveal moderate but significant aberrant IEL populations in patients who are also at high risk to develop EATL. Because of the high inter-observer rate, we recommend all patients suspected of RCD to undergo flow cytometric analysis of their duodenal biopsies.

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Chapter 6

Antibody titers against food antigens decrease upon a gluten-free diet, but are not useful for the follow-up of (refractory) coeliac disease

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Coeliac disease (CD) is an autoimmune disease characterized by intestinal mucosal damage as a consequence of a hypersensitivity to dietary gluten peptides. A gluten-free diet (GFD) reduces symptoms in the majority of patients within weeks to months.[1] However, mucosal recovery lags behind the clinical response.[2] There are indications that some of these histologically slow-responding patients have a poor prognosis.[3] Therefore, follow-up of mucosal recovery is required in CD patients. As biopsies are expensive and invasive, in clinical practice, the decrease in antibodies against tissue transglutaminase (TGA) is often used as an indicator of mucosal recovery. However, the use of only TGA as a marker may not be specific enough for the estimation of mucosal recovery.[4] Thus, other serum markers that can indicate intestinal mucosal recovery better than TGA are required. Potential serum markers for mucosal damage are serum antibodies against BSA and the baker's yeast Saccharomyces cerevisiae. Both antibodies against BSA (ABSA) and antibodies against S. cerevisiae (ASCA) are elevated in patients with uncomplicated CD, and ASCA have been reported to decrease on a GFD.[5-7] The rationale for using ASCA and ABSA as a marker for intestinal mucosal damage is that because of mucosal damage, the adaptive immune system is exposed to higher levels of dietary antigens and therefore more ABSA and ASCA may be produced. We therefore aimed to investigate the usefulness of ASCA and ABSA for the monitoring of intestinal mucosal recovery in CD patients on a GFD and in cladribine-treated type 2 refractory coeliac disease (RCD type 2) patients. Adult CD (n=35) and RCD type 2 (n=10) patients were selected from patients who visited our center from 2000 to 2011 with sera and histological data available at diagnosis and after treatment (Table 1). Biopsies were taken within 4 weeks before or after the date that the respective serum was taken. Immunoglobulin A (IgA)-deficient patients were excluded. The current study adhered to the guidelines set by the institutional ethical committee. CD was diagnosed according to current guidelines for adult CD.[8] RCD type 2 was diagnosed if patients, after having been on a strict GFD for at least 1 year, presented with an abnormal intraepithelial T-cell population together with reoccurring histological lesions.[9] The control group (n=27) included patients, who visited our hospital with sudden deafness, but did not show signs of autoimmune disease. Experienced pathologists evaluated the histology of duodenal biopsies according to the modified Marsh criteria.[10] The follow-up sera of CD patients with strict dietary adherence during the follow-up period (CD treated) were subdivided according to the presence of villous atrophy (Marsh 3; non-recovered; n=9) or the absence of villous atrophy (Marsh 0–2; recovered; n=26). Adherence to a GFD was confirmed by a dietitian. Patients in the non-recovered CD group with a follow-up of longer than 1 year after GFD were shown to be slow responders as they did recover (biopsy proven) at a later time point. RCD type 2 patients were treated with cladribine and subdivided into non-recovered (n=3) and recovered (n=7) on the basis of the Marsh classification as the CD group. TGA were measured by an in-house developed ELISA using human recombinant TG2 (Diarect, Freiburg, Germany) as described previously.[11] Sera containing more than 6 AU/ml TG2A were considered positive. Serum ASCA levels were measured using commercially available kits (QUANTA LiteTM ASCA IgA; Medical Technology Promedt Consulting GmbH, St. Ingbert, Germany) according to the manufacturer's recommendations. We measured ABSA by an in-house developed ELISA, using BSA (10 mg/ml, Albumin Fraction V; Roche Diagnostics

GmbH, Mannheim, Germany) coated 96-well plates (Nunc-MaxiSorp; Sanbio B.V., Uden, the Netherlands) and rabbit-anti-human-IgA conjugated with horseradish peroxidase (Dako, Glostrup, Denmark). Serum from a donor with a high concentration of BSA antibodies was used for the calibration curve. Before treatment, a wide range of ABSA and ASCA levels was found in both CD and RCD type 2 patients (Table 1). In the CD group, 45.7% of patients (n=16) had ABSA levels and 31.4% (n=11) had ASCA levels above the 95 percentile of the control group; in the RCD type 2 group, this was 60% (n=6) and 50% (n=5), respectively. The mean levels of both ABSA and ASCA were significantly higher in CD and RCD type 2 patients compared with the controls (all Pr0.05, Mann–Whitney U-test). Upon a GFD, ABSA levels significantly decreased in both CD patients who recovered histologically (Pr0.05, Wilcoxon signedrank test) and those who did not. In contrast, ASCA decreased significantly in patients who recovered histologically (Pr0.05, Wilcoxon signed-rank test), whereas the levels did not decrease significantly in patients who did not show histological recovery (Table 1). In RCD type 2 patients, neither ABSA nor ASCA showed a significant decrease in patients who recovered upon therapy (Table 1).

Table 1 *Patient characteristics and results.*

	Controls (N=27)	CD untreated (N=35)	CD treated / recovered (N=26)	CD treated / non-recovered (N=9)	RCD type 2 untreated (N=10)	RCD type 2 treated / recovered (N=7)	RCD type 2 treated / nonrecovered (N=3)
Age (years)	46±16	46±17	47±17	49±26	67±8	71±9	63±2
Sex, female [n %]	15 (55.6)	25 (71.4)	18 (69.2)	6 (66.7)	7 (70.0)	5 (71.4)	2 (66.7)
FU time (years)	NA	NA	1.3 [0.8-3.9]	1.2 [0.5-5.6]	NA	0.9 [0.3-1.7]	0.7 [0.7-1.0]
TGA-positive [n %]	0 (0)	35 (100)	5 (19.2)	4 (44.4)	0 (0)	0 (0)	0 (0)
ABSA (AU/ml)	30 [0.0-15.0]	12.5 [1.4-678][a]	6.4 [0.4-83.9][b]	5.9 [0.0-462]	18.7 [3.2-1237][a]	14.9 [0.0-508]	26.3 [14.2-439]
ASCA (AU/ml)	11.1 [6.8-31.8]	17.1 [5.0-232]	11.6 [05.4-35.7]	17.3 [6.3-244]	53.7 [7.8-207]	17.3 [5.8-230]	12.6 [8.0-33.6]

Normally distributed data are shown as mean±SD, categorical data as n (%), and nonparametrical data as median (5 percentile–95 percentile).
[a]P-value < 0.05 for comparison with controls.
[b]P-value < 0.05 for comparison with CD untreated.

Abbreviations:
ABSA anti-BSA antibody;
ASCA anti-Saccharomyces cerevisiae antibody;
CD coeliac disease;
FU follow-up;
NA not analyzed;
RCD refractory coeliac disease;
TGA transglutaminase antibodies.

Elevated levels of ABSA in the serum of CD patients before the GFD are in agreement with the findings of Rodriguez-Juan et al.[6], who investigated ABSA in CD. Here, we found that the levels of ABSA also significantly decrease on a GFD; however, the decrease is irrespective of the outcome of a GFD. Whereas ABSA have not been investigated in CD patients on a GFD before, ASCA have been shown to decrease in these patients.[5,7] ASCA have originally been investigated in Crohn's disease, with the rationale that they indicate enhanced intestinal permeability because of mucosal damage.[12] Accordingly, enhanced ASCA levels have been found in active CD, which is usually accompanied by increased intestinal permeability.[13] In that study, it was also reported that ASCA decreased significantly on a GFD and that patients with villous atrophy were more frequently ASCA positive than patients without villous atrophy. Here, we confirmed the findings of elevated ASCA in active CD and decreasing ASCA on a GFD. Furthermore, ASCA decreased particularly in CD patients who showed histological recovery. TGA titers, currently determined in the follow-up of CD to predict histological recovery, decrease rapidly upon initiation of a GFD, whereas recovery of mucosal damage may lag behind in a subset of patients.[4] We therefore tested whether ABSA and ASCA levels were associated with villous atrophy in patients who became TGA negative during the GFD (n=26) and found a similar decrease in ABSA and ASCA in both patients who recovered histologically and those who did not, indicating that as a diagnostic test to predict mucosal recovery in an individual CD patient, ABSA and ASCA have limited value. RCD patients are, per definition, TGA negative and alternative serum markers to predict mucosal recovery upon treatment would be of clinical significance. However, although both ABSA and ASCA are elevated in untreated RCD type 2 patients, neither test seems to be of diagnostic value as no significant decrease in the antibody levels was observed after successful treatment. Although the relatively small group sizes are a limitation of this study, the data clearly show that ABSA and ASCA are not useful to predict mucosal recovery in individual RCD patients in a diagnostic setting. To assess whether ABSA and ASCA levels are in general associated with intestinal damage in (complicated) CD, studies investigating larger group sizes are required.

We hypothesized that mucosal damage would enhance intestinal permeability and thus facilitate more contact of food antigens with the adaptive immune system, which then would lead to an enhanced production of antibodies against these antigens. However, our data show that intestinal permeability as determined by antibodies to food antigens is not necessarily related to villous atrophy. An alternative hypothesis could be that contact between food antigens and the mucosal immune system may be mediated by active transcytosis of antigens through enterocytes rather than because of the permeability caused by enterocyte apoptosis.[14] This is also suggested by the findings of van Schaik et al.[15], who have shown that ASCA can be elevated in Crohn's disease patients long before the diagnosis of the disease, which underlines that factors other than mucosal damage, such as possibly the local inflammatory milieu, may determine ASCA and ABSA levels. In conclusion, although ABSA and ASCA levels are elevated in CD and RCD type 2 patients and ASCA decrease differentially upon treatment, they are not useful for the follow-up of individual patients.

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Chapter 7

Serum parameters in the spectrum of coeliac disease: beyond standard antibody testing – a cohort study

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ABSTRACT

Background

Invasive techniques are still required to distinguish between uncomplicated and complicated forms of coeliac disease (CD).

Methods

We set out to investigate the potential use of novel serological parameters, including IL-6, IL-8, IL-17, IL-22, sCD25, sCD27, granzyme B, sMICA and sCTLA-4 in patients diagnosed with active CD, CD on a GFD, refractory coeliac disease (RCD) type 1 and type 2, and enteropathy-associated T-cell lymphoma (EATL).

Results

In both active CD and RCD type 1 and 2 elevated levels of the proinflammatory IL-8, IL-17 and sCD25 were observed. In addition, RCD type 2 patients displayed higher serum levels of soluble granzyme B and IL-6 in comparison to active CD patients. In contrast, no differences between RCD type 1 and active CD or RCD type 2 were observed. Furthermore, EATL patients displayed higher levels of IL-6 as compared to all other groups.

Conclusion

A series of novel serological parameters reveal distinctive immunological characteristics of RCD type 2 and EATL in comparison to uncomplicated CD and RCD type 1.

BACKGROUND

Coeliac disease (CD) is a chronic immune-mediated inflammation of the small intestine caused by a permanent state of intolerance to ingested gluten proteins affecting genetically susceptible individuals. Its hallmarks, lymphocytic infiltration of the lamina propria, expansion of the intraepithelial lymphocyte (IEL) population, hyperplasia of the crypts and atrophy of the villi are mediated by the interplay between an innate and adaptive immune response against gluten.[1] The complex of deamidated gliadin peptide interacting with the HLA-DQ2 and/or –DQ8 heterodimers on antigen presenting cells, is capable of activating the lamina propria T helper lymphocytes, thereby initiating antibody production and a gluten-specific pro-inflammatory type 1 T-cell response (Th1).[1] Th1 cells play an important role in pathogenesis of CD by secreting interleukin-2 (IL-2) that induces proliferation of T-lymphocytes and, in particular, by secreting the pro-inflammatory cytokine interferon-gamma (IFN-γ).[2] Furthermore, recent evidence has indicated that Th17 cells play a pathogenic role in CD.[3-5] In addition, interleukin-15 (IL-15) activates a cytotoxic response of intraepithelial lymphocytes through interferon-γ (IFNγ) release and upregulation of NKG2D. In combination with epithelial stress, the NKG2D ligand MHC Class I chain-related A (MICA) is upregulated by enterocytes.[6,7] The interaction of MICA and NKG2D induces enterocyte destruction by IELs. A gluten-free diet (GFD) results in mucosal recovery in the majority of patients, who are referred to as uncomplicated CD patients. However, a small subset of adult-onset CD patients fails to regain intestinal homeostasis after elimination of dietary gluten, or symptoms recur after initial response.[8] After careful evaluation of dietary compliance and exclusion of other possible disease-entities causing villous atrophy, these patients are diagnosed to suffer from refractory coeliac disease (RCD).[9] RCD is considered a complicated form of CD, and is divided into type 1, when patients lack an aberrant IEL population, or type 2 in which a substantial (>20%) aberrant IEL population is found in the small intestinal mucosa.[10,11] An aggressive type of lymphoma which carries a dismal prognosis, the enteropathy-associated T-cell lymphoma (EATL), is thought to arise from this aberrant IEL population. An interesting observation is that both aberrant IELs as well as EATL cells display a cytotoxic phenotype and contain high levels of granzyme B,[12,13] which could therefore serve as a marker for complicated CD. In consequence of a good response to immunosuppressive therapy, RCD type 1 patients have a better prognosis than RCD type 2 patients.[14-17] Therefore, early identification of CD patients developing RCD type 2 and/or EATL enables early intervention, which will likely reduce morbidity and mortality. Currently, antibodies against tissue transglutaminase (TGA), anti-endomysium (EMA) and deamidated gliadin peptides (DGPA) provide valuable and generally accepted serum parameters for the diagnosis and follow-up of uncomplicated CD. However, these antibodies are of no use in predicting and monitoring both types of RCD and EATL, implying that histological and flow cytometric analysis of duodenal biopsies are still required to distinguish between the uncomplicated and complicated forms of CD. Additional serum markers could potentially provide us with a minimal-invasive, easy applicable test without the need to perform a gastro-duodenal endoscopy. In addition, immunological markers in the peripheral blood could provide more insight in the similarities

and differences of the pathophysiology underlying the CD spectrum. Therefore, in the present study we evaluated several immunological and biochemical parameters in sera from the five stages of CD, including active CD (ACD), CD on GFD, RCD type 1 and 2, and EATL, for their ability to differentiate between complicated and uncomplicated forms, and secondly, to gain insight in the pathophysiological relations between these disease entities. For this purpose, we analysed serum levels of the inflammatory cytokines IL-6, IL-8, IL-17and IL-22, the T-cell activation factors soluble (s)CD25 (IL2R-alpha) and sCD27, the T-cell dysregulation factor sCTLA-4, shown previously to be up-regulated in different autoimmune diseases, the cytotoxic T-cell parameter granzyme B, and sMICA, previously shown to be associated with the presence of epithelial stress and malignancies.

METHODS

A retrospective cohort study was conducted at a tertiary referral centre for coeliac disease in The Netherlands. Patients previously diagnosed with (complicated) CD in the VU medical center were identified and included in our study when stored serum samples at time of diagnosis were available. Overall, 92 blood samples collected for diagnostic purposes between 1997 and 2010 were included. Serum levels of a substantial number of immunological markers were determined in the five different subsets of CD. In addition, results from several biochemical parameters of this cohort at time of diagnosis were collected from the electronic patient file in our centre.

Patients

CD diagnosis was based on the ESPGHAN guidelines.[18] All patients included in the active CD group had positive EMA and/or TGA, and histological abnormalities grade III according to the modified Marsh classification consisting of intraepithelial lymphocytosis, crypt hyperplasia and some degree of villous atrophy.[19] Furthermore, serum samples of these patients at time of an inactive phase of CD were collected. Remission of disease (GFD group) was defined by the disappearance of one or both CD antibodies upon a GFD, and if a gastroduodenal endoscopy with subsequent collection of biopsies was performed during follow-up, normalisation of mucosal abnormalities (Marsh 0 or I) was required. Patients included in the RCD group had persisting or recurring symptoms and small intestinal villous atrophy, despite strict adherence to a GFD for at least one year (assessed by a dietitian and negative TGA/EMA). The clinically validated cut-off value of more than 20% of the IELs expressing an aberrant phenotype (surface $CD3^-$, but cytoplasmic $CD3^+$) as detected by flow cytometric analysis was used to distinguish RCD type 1 and type 2.[10] In total, 26 paired serum samples of CD patients at time of disease activity (ACD group) and after normalisation of the CD associated antibodies upon a GFD (GFD group), and of an additional 40 patients with complicated CD at diagnosis were included. The latter group consists of 12 RCD type 1, 16 RCD type 2 and 12 EATL patients. All procedures were in accordance with the regulations of the medical ethics committee, and all patients declared their informed consent to store and use their blood samples collected for regular diagnostics for further research.

Serum parameters: Enzyme linked immunosorbent assay (ELISA)

Levels of cytokines IL-6, IL-8, IL-17 and IL-22 were determined in serum using commercial ELISA kits (Pelikine-compact™, Sanquin, Amsterdam, The Netherlands), according to the manufacturer's instructions. Levels of soluble CD25 (sCD25), soluble CD27 (sCD27), soluble CTLA-4 (sCTLA-4), soluble MICA (sMICA) and granzyme-B were determined in serum using a specific commercial ELISA kit (Diaclone, Besancon, Cedex, France), according to the manufacturer's instructions.

Biochemical parameters

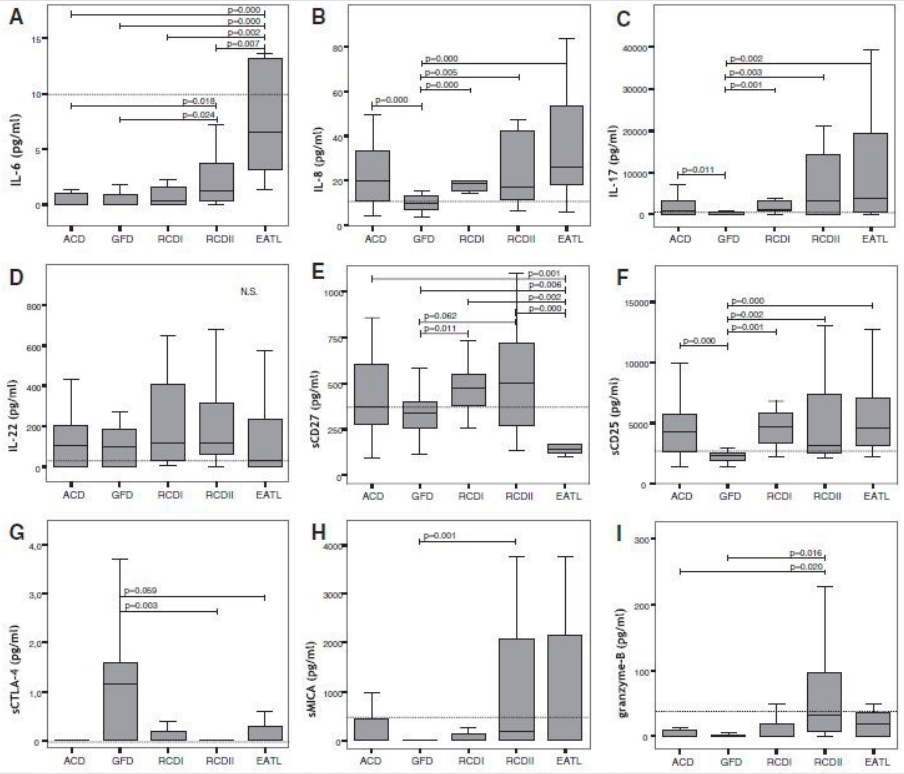
The concentration of C-reactive protein (CRP; g/L), erythrocyte sedimentation rate (ESR; mm per 1 h), leukocyte count (WBC; 10E9/L), albumin (g/L) and haemoglobin (Hb; mmol/L) were extracted from the hospital patient file for all patients included at time of diagnosis.

Statistical analysis

Data were analysed and plotted using SPSS software (SPSS Inc. Chicago, Illinois, USA), using non-parametric tests as most variables examined in this study did not appear to be normally distributed. A Wilcoxon signedrank test was used for pairwise comparison of the variability of immunological and biochemical parameters among the ACD and GFD group. The latter groups were individually compared to the complicated forms of CD by using the Kruskal-Wallis non-parametric test to identify possible serum and biochemical differences in the spectrum of CD. A receiver operating characteristic (ROC) curve was made of all significantly different parameters to represent the trade-off between the false negative and false positive rates. As a considerable number of markers were determined, the level of significance was set at highly significant ($p < 0.001$) and moderately significant ($0.001 < p < 0.05$).

RESULTS

Table 1 shows the characteristics of the five subsets of CD. Serum samples of 26 ACD patients at time of diagnosis and after remission of disease on a GFD were included. In all patients CD associated antibodies reverted to negative upon a GFD, and in 62% (16/26) a biopsy was taken which revealed mucosal healing (Marsh 0/I) in all those evaluated. Overall, 12 RCD type 1, 16 RCD type 2 and 12 EATL patients were included. Significant differences were found for cytokine profiles between the five subsets of CD, as described in more detail below (Figure 1A-I).



The dotted line corresponds with the upper level of the normal range.

Abbreviations:

CD coeliac disease;
ACD active coeliac disease;
GFD response upon a gluten-free diet;
RCD 1 refractory coeliac disease type 1;
RCD 2 refractory coeliac disease type 2;
EATL enteropathy-associated T-cell lymphoma.

Figure 1 *Concentration of serum parameters in the different subsets of the CD spectrum IL-6* ***(A)*** *; IL-8* ***(B)****; IL-17* ***(C)****; IL-22* ***(D)****; sCD25* ***(E)****; sCD27* ***(F)*** *; sMICA* ***(G)****; sCTLA-4* ***(H)****; granzyme B* ***(I)****.*

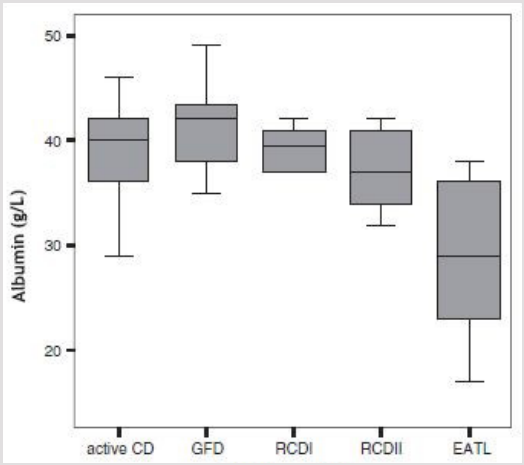
Table 1 *Characteristics of the different groups in the spectrum of coeliac disease.*

		SPECTRUM OF CD				
		aCD (n=26)	**GFD (n=26)**	**RCD 1 (n=12)**	**RCD 2 (n=16)**	**EATL (n=12)**
Age at CD (yr)	Median, (SD;range)	42.5 (16; 21-76)	45 (15; 21-77)	50.5 (16; 19-69)	61.5 (13; 27-74)	61.5 (7; 48-66)
Age at RCD	Median, (SD;range)	-	-	59 (11.5; 35-74)	65 (10; 47-77)	64 (8; 51-72)
Age at EATL	Median, (SD;range)	-	-	-	-	65 (6; 51-74)
HLA-staus	DQ2 heterozygous	10	7	8	7	3
	DQ8 heterozygous	9	13			3
	DQ2/8 heterozygous	3	2	1	3	2
	DQ2 homozygous	4	4	3	6	4
Marsh	0		12			2
	I		4			
	II					1
	IIIA	11		8	9	5
	IIIB	5		3	4	3
	IIIC	10		1	3	1
	ND		10			
EMA	Negative (-)		26	12	15	7
	Dubious (+/-)				1	2
	Weak positive (+)	3				
	Positive (++)	11				0
	Stronglypositive(+++)	11				2
	ND	1				1
tTGA	Negative		7	12	14	6
	Dubious				2	2
	Weak positive	8				1
	Positive	6				1
	Strongly positive	9				1
	ND	3	19			1
Aberrant IELs (%)	Mean (SD;range)	-	-	4,0 (6.0; 2-19)	64 (23; 20-96)	7 (32; 1-87)
EATL type	Primary	-	-	-	-	4
	Secondary					8

Abbreviations:
ACD active coeliac disease patients;
GFD coeliac disease patients on a gluten-free diet;
RCD 1 and 2 refractory coeliac disease type 1 and 2;
EATL enteropathy-associated T-cell lymphoma;
EMA-A endomysial antibodies;
TGA tissue transglutaminase antibodies;
IELs intraepithelial lymphocytes.

Active CD versus GFD

Serum levels of the inflammatory chemokine IL-8 (p = 0.00) and the T-cell activation factor sCD25 were higher in active CD patients than in patients in remission on a GFD (p = 0.00, highly significant) as well as the Th-17 lineage-defined cytokine IL-17 levels (p = 0.011, moderately significant). Serum levels of IL-6, IL-22, sCD27, sMICA, granzyme-B and sCTLA-4 were not significantly elevated in the ACD group. In addition, levels of CRP, ESR, albumin and leukocyte counts were similar (data not shown).



The concentration of albumin is significantly higher in both the active CD (p=0.003) and the GFD (p=0.000) subset compared to the complicated forms of CD. In EATL patients clearly lower concentrations compared to both RCD type 1 and type 2 (p= 0.003 and p=0.037, respectively) were observed.

Abbreviations:
CD coeliac disease;
ACD active coeliac disease;
GFD response upon a gluten-free diet;
RCD 1 refractory coeliac disease type 1;
RCD 2 refractory coeliac disease type 2;
EATL enteropathy-associated T-cell lymphoma.

Figure 2 *Concentration of albumin in the different subsets of the CD spectrum.*

Uncomplicated CD versus RCD

The serum levels of IL-8, IL-17 and sCD25 in RCD type 1 and type 2 were comparable to those in the ACD group, however, significantly lower levels were observed in the GFD group. Moreover, in comparison to both ACD and GFD patients, RCD type 2 patients showed increased levels of granzyme-B (p = 0.020; p = 0.016, both moderately significant) and IL-6 (p = 0.018; p = 0.024, both moderately significant), respectively. Furthermore, serum levels of soluble CTLA-4 were lower in RCD type 2 patients than those in remission upon a GFD (p = 0.003, moderately significant). Similar IL-22 serum levels were found in uncomplicated CD and RCD type 1 and type 2. Comparison of the inflammatory parameters CRP, ESR and leukocyte count did not reveal significant differences between uncomplicated CD and RCD type 1 and type 2. The concentration of albumin was lower in both RCD subsets when compared to ACD (p = 0.003, moderately significant) and GFD (p = 0.000, highly significant; Figure 2).

The RCD complex

None of the markers tested was able to distinguish RCD type 2 from RCD type 1. Moreover, no significant differences in levels of albumin or inflammatory parameters CRP, ESR and leukocyte count were observed.

EATL versus ACD and RCD

The highest serum levels of IL-6 were observed in the EATL group, were higher ($p = 0.000$, highly significant) as compared to the ACD and GFD groups, as well as higher ($p = 0.002$; $p = 0.007$, moderately significant) than RCD type 1 and type 2, respectively. Moreover, serum levels of IL-6 were clearly elevated in EATL over RCD type 2 with an AUC of 0.82 [95% CI: 0.649-0.971]. IL-6 levels in EATL patients tended to correlate (0.45, $p = 0.08$) with CRP levels, but not with IL-17 levels. Furthermore, serum levels of sCD27 were decreased (moderately significant) in EATL patients as compared to all other groups, except RCDII in which (highly) significant ($p = 0.000$) differences were found). Nevertheless, ROC analysis resulted in a very low AUC. In addition, similar levels of sMICA, granzyme-B and sCD25 were measured in EATL and RCD type 1 and type 2. However, the serum albumin concentration in EATL patients was lower (moderately significant) than in ACD and both types of RCD (Figure 2).

DISCUSSION

Currently, physicians are unable to predict who will develop complicated CD. As invasive techniques are still required to differentiate between uncomplicated and complicated forms of CD, the purpose of the present analysis was to evaluate the ability of markers in peripheral blood to distinguish the various CD subsets. By doing so, it may also provide insight in the potential differences in underlying immunopathomechanisms of these disease subsets. The results could be biased by cytokine production from different cellular sources in the peripheral blood, and therefore may not exactly mimic the intestinal inflammation.[20] However, other investigators have previously shown that blood cytokine profiles do reflect intestinal mRNA expression in active CD patients.[2,21-23] Care must be taken in case our findings are extrapolated to other age groups, as it cannot be excluded that normal values vary over age.

Active CD versus GFD

Our results are in keeping with previous studies reporting on serum cytokine levels in ACD patients, that showed up-regulation of IL-8,[20,24] a chemokine produced by macrophages, epithelial as well as endothelial cells that attracts leukocytes to a site of inflammation. The same accounts for the observed elevated levels of sCD25, that is cleaved off from the membranous CD25 (IL-2R-alpha) during T-cell activation.[25-27] Furthermore, the increased levels of the pro-inflammatory cytokine IL-17 observed in the current study, supports the view that Th17 cells that produce this particular cytokine are involved in several auto-immune diseases and are suggested to have a pathogenic role in CD.[3-5] In contrast to the (pro-) inflammatory environmental characteristics observed in this analysis and to previous reports,[20,26,28] the levels of IL-6, illustrative of an acute phase response and a potent inducer of the Th17 pathway[29], were low and similar in both uncomplicated CD subsets. A clear explanation for these findings is lacking, especially as strongly increased levels were found in RCD type 2 and EATL patients. Another cytokine involved in gut inflammation is IL-22 that is produced by Th17 and/or Th22 cells, where it is currently believed to exert regulatory functions.[30,31] Elevated levels of IL-22 have been found in the mucosa of Crohn's disease[32], but its role in CD is yet unclear.[3,33] Nevertheless, our results failed to reveal a correlation with IL-22 serum levels and disease activity or disease entity. sCD27 serum levels are increased in T-cell mediated diseases, including some auto-immune disorders.[34,35] However, the sCD27 levels were normal in ACD. This in contrast to the previously reported increased production of another lymphocyte activation marker sCD25. This dissimilarity is remarkable as in SLE patients sCD25 and sCD27 levels are strongly correlated during the whole disease course.[36]

The RCD complex

Although, by general consent, RCD type 1 and 2 are considered two related disease entities within the spectrum of CD, it remains unclear if both diseases share a similar pathogenesis responsible for the gluten independent inflammation. In keeping with the current opinion, our results showed in both types of RCD similar inflammatory characteristics and T-cell activation

based on serum levels of IL-8, IL-17, IL-22 and sCD25, respectively. On the other hand, a transition from RCD type 1 to RCD type 2 has only been reported sporadically.[16] Theoretically, granzyme B is a suited parameter to differentiate between RCD type 1 and 2, since aberrant IELs are clearly cytotoxic and express high amounts of intracellular granzyme-B.[12] Although increased levels of soluble granzyme-B were observed in RCD type 2 patients, these were not significantly higher than in RCD type 1 patients.

RCD versus uncomplicated CD

Currently, it is unknown to what extent the gluten independent inflammation as generally observed in RCD evolved from and/or differs from the gluten induced inflammation in active CD. In this study, the proinflammatory T-cell response, including IL-8, IL-17, sCD25, in both types of RCD and ACD patients shows resemblance, with exception of evidently increased IL-6 levels in RCD type 2 over active CD. This finding suggest a higher inflammatory state in RCD type 2 than in ACD, however, similar levels of the inflammatory parameters CRP, ESR and leukocyte count were observed. In line with the lack of intestinal inflammation in patients adhering to a GFD, the pro-inflammatory response was significantly lower as compared to the complicated forms of CD. Interestingly, in comparison to ACD patients, RCD type 2 patients displayed a distinctive cytotoxic T-cell activation profile based on elevated serum levels of granzyme B. The levels of these parameters observed in RCD type 1 patients did not differ from either ACD or RCD type 2 patients.

Monitoring EATL development

RCD type 2 patients carry a high risk to develop an EATL, yet, so far no serum parameters for EATL development have been identified, including efforts in the present analysis. Based on the fact that EATL cells contain large amounts of granzyme B,[13] these levels were measured in the peripheral blood, but EATL patients did not contain higher levels than active CD or RCD patients. Furthermore, elevated levels of sCD27[37] and sCD25[38] have been suggested to be associated with tumour burden in some lymphoid neoplasia. Neoplastic lymphoid cells in non-Hodgkin lymphoma express CD27 and are considered responsible for the increased sCD27 production.[37] The current analysis failed to show increased sCD27 levels in EATL patients and thereby suggests that EATL regards a distinct type of lymphoma and does not aid in the identification of this particular lymphoma. In contrast to a previous study showing elevated sCD25 levels in EATL,[39] we found comparable levels of sCD25 in EATL, both types of RCD, and ACD. The same accounts for sMICA, that is cleaved from membranous MICA and has been shown to impair NKG2D mediated tumor surveillance in epithelial tumors,[40] as well as it has shown potential as a prognostic parameter in hematopoietic malignancies since increased levels of sMICA were found in leukemia patients.[41] The only marker that distinguished EATL from all other groups was IL-6 and its levels correlated with CRP levels, indicating a more severe acute inflammatory response in EATL patients that is distinctive from the other subsets of CD. In accordance with our data, an association with IL-6 levels and survival in Hodgkin lymphoma has been recognized almost twenty years ago.[42]

Cytokine levels in comparison with other gastro-intestinal diseases

Our data suggests that complicated CD is accompanied by a higher pro-inflammatory state as compared to uncomplicated CD. To provide insight in the extend of this inflammation, it can be compared to the cytokine profile in other (small) intestinal disease, such as Crohn's disease. Not only have elevated serum levels of IL-6 been reported in this disease, but these levels appear even higher than in complicated CD.[43] On the other hand, IL-6 and IL-8 serum levels in Helicobacter Pylori infected patients with peptic ulcer disease are not elevated.[44]

Clinical implication and potential application

Taken together, for daily clinical practice the results of this analysis suggest that apart from detection of CD associated antibodies and duodenal biopsies, other variables including IL-8, sCD25 and possibly IL-17, might be helpful in monitoring inflammatory disease status and differentiating between patients on a strict GFD and those diagnosed as having RCD type 1 and type 2. In case CD specific antibodies remain mildly elevated, which is not rare in RCDI-II patients, serum levels of granzyme-B could possibly serve as an additional markers to distinguish ACD from RCD type 2, and will enable early intervention. However, the currently accepted diagnostic work-up of RCD remains required, pending prospective serum studies including larger series of patients.

CONCLUSION

In conclusion, both types of RCD are characterised by an ongoing and/or recurring inflammatory disease status showing great resemblance to that observed in ACD despite strict adherence to a GFD, yet differentiates itself by elevated serum IL-6 concentrations in RCD type 2. Furthermore, in addition to this increased pro-inflammatory profile, RCD type 2 reveals a distinctive cytotoxic T-cell activation profile as compared to ACD based on elevated levels of granzyme-B, whereas RCD type 1 does not. Although no EATL-specific or -associated immunological parameters were found in this study, our ongoing efforts may identify relevant markers. Further research will also address the prospective and diagnostic value of the serum variables identified is this study in order to expand the clinical application of (R)CD serology beyond standard autoantibody testing.

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PART III

PATHOGENESIS

Chapter 8

Origin and immunophenotype of aberrant IEL in RCD type 2 patiënts

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ABSTRACT

Objectives

Aberrant intraepithelial lymphocytes (IELs) are the hallmark of refractory coeliac disease type 2 (RCD type 2) and considered a premalignant cell population from which aggressive enteropathy-associated T-cell lymphoma (EATL) can evolve. The aim of this study was to gain further insight in the origin and characteristics of aberrant IELs by analysing T-cell receptor (TCR) rearrangements, and by immunophenotypic analysis of aberrant IELs.

Design

Duodenal biopsies from 19 RCD type 2 patients and three RCD type 2 cell lines were analysed for the presence of TCR delta, gamma, and beta rearrangements. In addition, IELs isolated from biopsies derived from RCD type 2 patients were phenotypically analysed.

Results

Aberrant IELs showed an upregulated expression of granzyme B and decreased expression of PCNA. TCR rearrangements in the aberrant IEL population in biopsies of RCD type 2 patients were heterogenic, which is most likely due to a variation in maturity. Similarly, RCD type 2 cell lines displayed a heterogenic TCR rearrangement pattern.

Conclusion

Aberrant IELs originate from deranged immature T-lymphocytes and display clear differentiation to a cytotoxic phenotype. Aberrant IELs displayed different stages of maturity between RCD type 2 patients, of which only the patients harbouring the most mature aberrant IEL population developed an EATL.

INTRODUCTION

Coeliac disease (CD) is a common small intestinal enteropathy induced by dietary gluten proteins as well as other undefined environmental factors, affecting genetically predisposed individuals of all ages. A permanent state of intolerance to gluten-containing food leads to a chronic auto-immune mediated inflammatory response with subsequent remodelling of the proximal small bowel mucosa and nutrient malabsorption.[1] Withdrawal of dietary gluten usually leads to prompt healing of the damaged small-intestinal mucosa and improvement of nutrient absorption.

Although a substantial group of adult-onset CD patients lacks histological recovery after 2 years on a gluten-free diet (GFD) only a small subgroup of patients,[2,3] especially those diagnosed above the age of 50 years, develop primary or secondary resistance to a gluten-free diet (GFD) with intestinal villous atrophy and persisting or reoccurring symptoms of malabsorption.[4] After evaluation of diet-compliance by a dietitian and exclusion of other underlying diseases known to cause villous atrophy, subjects are considered to suffer from refractory coeliac disease (RCD).[5] Based on the immunophenotype of intraepithelial lymphocytes (IELs), RCD can be subdivided into type 1 lacking a substantial aberrant IEL population ($CD3^-$ $CD45^+$ $CD103^+$ $CD7^+$ $CD4^-$ $CD8^-$ $cytCD3^+$ cells) and type 2 in which an aberrant IEL population is present.[6] The distinction between RCD type 1 and type 2 is defined by a clinically validated cut-off of 20% aberrant IELs.[7]

As a consequence of a generally good response to immunosuppressive therapy, RCD type 1 has a less dismal prognosis compared to patients suffering from RCD type 2, reflected in a 5-year survival rate of approximately 90% and 44-58%, respectively.[8-11] More importantly, approximately 40-50% of all RCD type 2 patients develop an aggressive enteropathy-associated T-cell lymphoma (EATL), which is considered to arise from the clonal expansion of the premalignant aberrant IEL population.[12-14] EATL is one of the main causes of death in RCD patients, due to its aggressive nature and unresponsiveness to currently available therapies.[8-11] Despite standardized treatment, one half of the RCD type 2 patients develop an EATL whereas the other half does not, which could indicate that aberrant IEL populations between patients are heterogeneous and might be accompanied with a variable risk to develop an EATL. Even though RCD type 2 and EATL patients are associated with HLA-DQ2 homozygosity,[15] there currently are no histopathological or immunophenotypic features have been identified that have a prognostic value in the evolution of aberrant IEL into an EATL. Therefore it is of utmost importance to gain more insight in the origin and characteristics of aberrant IELs in RCD type 2, which will enable a better identification of high risk patients and the development of new therapeutic options.

Recently, elegant work addressing the expansion and function of aberrant IELs has been performed,[16,17] nevertheless, the exact role of aberrant IEL in the mucosa of the small intestine still remains unclear. Furthermore, the cells from which monoclonal aberrant IELs originate is currently under debate. Although aberrant IELs found in RCD type 2 do not express the T-cell lineage specific surface CD3-TCR complex, these cells do contain cytoplasmatic CD3 antigen and display T-cell receptor (TCR) rearrangements, indicative of T-cell lineage commitment. It has been suggested that the TCR-CD3 complex is internalized due to overstimulation of IELs,

implying that aberrant IELs originate from mature TCR^+ IELs.[13] More specifically, it is hypothesised that these cells derive from γδ T-lymphocytes based on the observed inversed correlation between aberrant IELs and γδ T-cells in RCD type 2.[18,19]

Alternatively, a small, unique $CD3^-$ $CD7^+$ population considered to be NK / T-cell precursors, which is found in the intestine of healthy individuals, is suggested to represent the physiological counterpart of aberrant IELs.[20-22] The presence of an immature lymphoid precursor population in the gut mucosa, which could hypothetically serve as origin for aberrant IELs, is emphasized by the ongoing extrathymic maturation of T-cells in the intestinal mucosa throughout life.[23]

Therefore, in this study we isolated lymphocytes from duodenal biopsies collected from RCD type 2 patients and compared the expression of markers representing activation, proliferation, DNA-repair and lymphocyte development on aberrant lymphocytes to the expression on normal lymphocytes within the same patient. To elucidate the origin of aberrant IELs, we assessed these biopsies for the presence of TCR delta (TCRD), gamma (TCRG) and beta (TCRB) gene rearrangements. In addition, extensive analysis of TCR gene rearrangements in RCD type 2 cell lines was performed.

PATIENTS AND METHODS

Patients

RCD type 2 patients included in this study visited the out-patient department of gastroenterology at the VU University Medical Centre, Amsterdam, The Netherlands for diagnostic work-up or regular follow-up. The diagnosis of RCD type 2 was based on persisting or recurring symptoms and small intestinal villous atrophy after a former good response despite strict adherence to a gluten-free diet for at least 1 year. Furthermore, the clinically validated cut off value of 20% aberrant IELs as detected by flow cytometry was predominantly used to distinguish RCD type 1 and type 2.[7] A lower percentage of aberrant T-cells was allowed in the presence of ulcerative jejunitis.

Small intestinal biopsies

During upper gastrointestinal endoscopy multiple large spike forceps biopsies were taken from the second part of the duodenum. For TCR rearrangement analysis, 1-2 biopsy specimens were stored in liquid nitrogen until analysis. For flow cytometric evaluation, 6 biopsy specimens were collected from various locations in the duodenum, from which IELs were isolated and pooled before immediate analysis. All biopsy specimens were obtained for diagnostic purposes and the procedures were in accordance with the ethical guidelines of our institution.

Cell lines and cell culture

RCD cell lines P1, P2 and P3 were isolated from duodenal biopsies of RCD type 2 patients as previously described.(24) In short: biopsy specimens were treated with 1mM dithiothreitol (Fluka, Buchs, Switzerland) and 0.75 mM ethylenediaminetetraacetic acid (Merck, Darmstadt, Germany) after which the biopsy was cultured in Iscove's modified Dulbecco's medium (IMDM, Lonza, Verviers, Belgium) supplemented with 10% normal human serum (NHS) and 10 ng/ml IL-15 (R&D systems Europe, Abingdon, UK). Released cells were propagated on IMDM containing 10% NHS and 10ng/ml IL-15 and restimulated approximately every 4 to 5 weeks with 1 µg/ml phytohemagglutinin, 10 ng/ml IL-15 and 1 x 10^6/ml irradiated allogenous peripheral blood mononuclear cells as feeder cells. A TCR-$CD3^+$ $CD4^+$ T-cell clone isolated from a duodenal biopsy of a CD patient was maintained in IMDM containing 10% NHS supplemented with 10 ng/ml IL-15 and 20 Cetus units/ml IL-2 (Proleukin, Chiron corporation, Emeryville, CA) and restimulated every 2 weeks.

Flowcytometric analysis

Multiparameter flowcytometric immunophenotyping was performed on IEL suspensions, isolated as previously described.[7] Briefly, biopsies were vigorously shaken at 37°C for 60 min in PBS supplemented with 1 mM DTT (Fluka BioChemika, Buchs Switzerland) and 1 mM EDTA (Merck, Darmstadt Germany). The released IELs were washed twice with PBS supplemented with 0.1% BSA (Roche Diagnostics) and subsequently stained for 30 min on ice, with fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridinin chlorophyll protein (PerCP) and

allophycocyanin (APC)-labeled monoclonal antibodies directed against CD3, CD4, CD5, CD7, CD8, CD16+56, CD19, CD25, CD30, CD34, CD45, CD45RA, granzyme B, HLA-DR, Ki-67, NKG2D, PCNA (all from BD Biosciences, San Jose, CA), CD52 (Serotec, Düsseldorf, Germany), terminal deoxynucleotidyl transferase (TdT) and CD1a (Glostrup, Denmark), CD103 (IQ products, Groningen, Netherlands), and IL15R-alpha (eBioscience, Hatfield, United Kingdom). Cytoplasmic staining of CD3 was performed after cell permeabilisation by Cytofix/CytoPerm Plus (BD Biosciences), according to the manufacturer's instructions. Nuclear staining of Ki-67 and PCNA was performed after cell permeabilisation by eBioscience Cell Fixation/Permeabilisation (eBioscience), according to the manufacturer's instructions. Stained cells were washed with PBS containing 0.1% bovine serum albumin (BSA, Sigma) and analysed on a standard 4-color flowcytometer (FACSCalibur, BD Biosciences). The data were analysed using Cellquest software (BD Biosciences). Care was taken to analyse only viable cellular events based on light scatter properties.

Both aberrant IELs (sCD3$^-$ CD45$^+$ cytCD3$^+$) and normal IELs (sCD3$^+$ CD45$^+$ cytCD3$^+$) were reported as percentage of the total number of CD45$^+$ (lymphocytic) cells.[3,4] The expression level of the aforementioned markers on aberrant lymphocytes was compared to that of IELs with a normal immunophenotype within each individual and reported in percentage and-or mean fluorescent intensity (MFI) index. For all markers corresponding isotype controls were used.

PCR-based analysis of TCRD, TCRG and TCRB gene rearrangements

TCRD, TCRG and TCRB gene rearrangements were assessed by multiplex PCR as previously described and resulting PCR products were further evaluated by GeneScan analysis.[25]

Southern blot

Southern blot analysis of TCRB and TCRD genes was performed according to earlier published protocols, using TCRDJ1, TCRBJ1 and TCRBJ2 probes.[26,27]

Statistical analysis

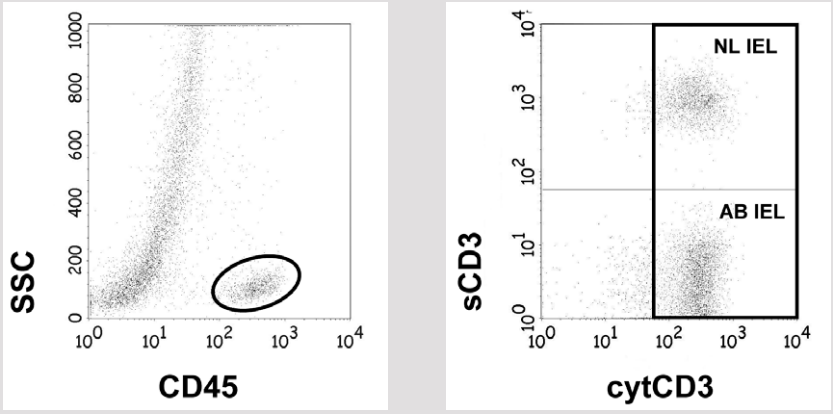
Data were analysed in SPSS software (SPSS Inc. Chicago, Illinois, USA). Flowcytometry data were analysed with the non-parametric MannWhitney test to test for differences between aberrant lymphocytes and its normal counterpart. A p-value ≤ 0.05 was considered significant.

RESULTS

Flowcytometric analysis

An extensive panel of surface- and intracellularly expressed markers was evaluated by multiparameter flowcytometry. Overall, immunophenotyping of duodenal biopsies was performed during follow-up in 16 RCD type 2 patients characterised by a median aberrant IEL percentage of 58.5% (range 21%- 97% ; Table 1). All subjects except one were treated with cladribine and three were subsequently treated with high dose chemotherapy followed by autologous haematopoietic stem cell transplantation as previously described.[28,29] In addition, 6 subjects visited the department for follow-up more than once. In these patients, repetitive flow cytometry analysis showed almost identical results regarding the immunophenotype of aberrant and normal IELs (data not shown), indicating stability of the observed aberrant IEL phenotype. Table 3 shows an overview of all markers analysed and their respective expression by both aberrant and normal IELs.

All IELs showed strong expression of CD45 and low to intermediate forward and sideward scatter, indicating a relatively homogeneous population of lymphocytic cells (Figure 1A). In keeping with the generally accepted aberrant IEL immunophenotype found in RCD patients, this population expressed cytoplasmic CD3 but lacked expression of surface CD3 (Figure 1B). In addition, these cells expressed CD7 (Figure 2A). Expression of the early T-cell development-associated markers terminal deoxynucleotide transferase (TdT), CD1a and CD34 was not detected (not shown). Aberrant IELs did not show expression of CD5 (Figure 2B). Interestingly, 30% of the normal IEL population lacked CD5 expression, in contrast to the uniform expression of CD5 by peripheral T-cells .



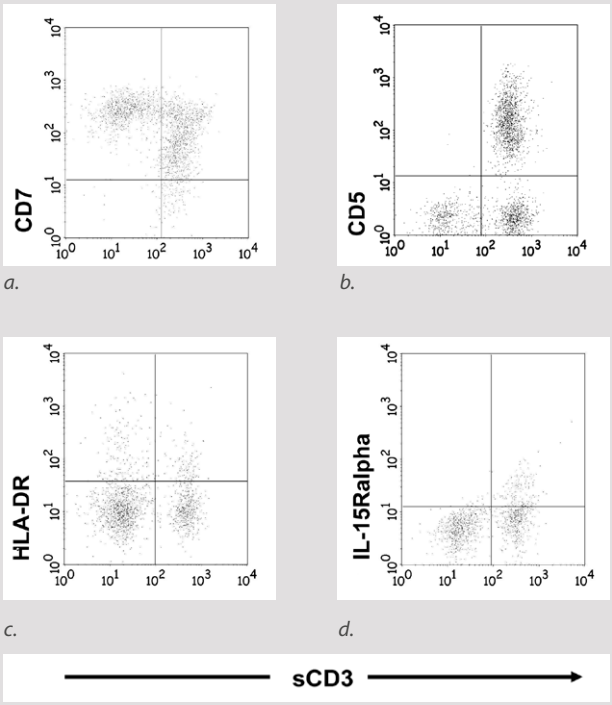
a. *b.*

*Gating strategy to identify aberrant and normal IELs. Cells with a strong CD45 expression and low to intermediate forward and sideward scatter were selected **(a)**, after which IELs expressing intracellular CD3 expression were used for further studies **(b)**.*

Figure 1.

Table 1 Patient characteristics.

patient	analysis	sex	Age RCD type 2	HLA-DQ	Dx_ Marsh	Dx_ aberrant IELs (%)	TCR_ aberrant IELs (%)	FACS_ Marsh**	FACS_ aberrant IELs (%)	EATL develop-ment	patient	analysis
1	TCR	F	64	DQ2+/ DQ8+ heterozygous	IIIB	50	27	N.D.	N.D.	No	No	N.D.
2	TCR	M	50	DQ2 homozygous	IIIA	67	9; UJ	N.D	N.D	No	No	N.D
3	TCR + FACS	M	68	DQ2 homozygous	IIIB	87	88	0	91	No	No	2-CdA; auSCT
4	TCR + FACS	M	67	DQ2 heterozygous	IIIA	61	61	0	58	No	No	2-CdA
5	TCR + FACS	F	66	DQ2 heterozygous	IIIC	53	53	0	45	No	No	2-CdA
6	TCR	F	62	DQ2 heterozygous	IIIB	50	50	N.D.	N.D.	No	2-CdA; auSCT	N.D.
7	TCR + FACS	F	52	DQ2 homozygous	IIIB	87	87	IIIA	88	No	No	2-CdA
8	TCR + FACS	M	72	DQ2 homozygous	IIIA	59	59	0	80	No	No	2-CdA
9	TCR + FACS	F	67	DQ2 heterozygous	IIIC	55	71	IIIA	61	No	No	2-CdA
10	TCR	F	67	DQ2 heterozygous	IIIB	11	11; UJ	N.D.	N.D.	No	No	N.D.
11	TCR + FACS	M	62	DQ2 heterozygous	IIIA	75	75	IIIA	59	No	No	2-CdA
12	TCR	M	78	DQ2 homozygous	IIIA	74	74	N.D.	N.D.	No	No	N.D.
13	TCR + FACS	M	77	DQ2 heterozygous	IIIA	92	92	IIIA	97	No	No	2-CdA
14	TCR	M	57	DQ2 homozygous	IIIB	78	78	N.D.	N.D.	No	2-CdA	N.D.
15	TCR + FACS	F	70	DQ2 heterozygous	IIIA	37	37	IIIA	21	No	No	No
16	TCR	M	59	DQ2 homozygous	IIIA	95	95	N.D.	N.D.	Yes	No	N.D.
17	TCR	F	61	DQ2 heterozygous	IIIC	59	59	N.D.	N.D.	Yes	2-CdA	N.D.
18	TCR	F	64	DQ2 homozygous	IIIC	88	88	N.D.	N.D.	Yes	No	N.D.
19	FACS	F	64	DQ2 heterozygous	IIIB	21	N.D.	IIIA	21	No	N.D.	2-CdA
20	FACS	F	56	DQ2 heterozygous	IIIB	65	N.D.	0	76	No	N.D.	2-CdA; auSCT
21	FACS	F	47	DQ2 heterozygous	IIIC	92	N.D.	0	65	No	N.D.	2-CdA; auSCT



a. *b.* *c.* *d.*

Surface expression of CD7 ***(a)****, CD5* ***(b)****, HLA-DR* ***(c)*** *and IL-15R-alpha* ***(d)*** *on aberrant IELs (Ab IEL) and normal IELs (NL IEL) derived from case 5, case 19, case 13 and case 5 (Figure 1) illustrate representative data from individual patients.*

Figure 2.

Significantly more aberrant lymphocytes than normal IELs expressed intracellular granzyme B, illustrating a cytotoxic phenotype ($p<0.05$) (Figure 3A-C; Table 2). No expression of CD30, or its ligand CD153 was observed on aberrant cells, thereby confirming the absence of an EATL.[30] However, we did find expression of CD45RA on both normal and aberrant IELs, which is known to be associated with cytotoxic activity in antigen experienced T-cells. Furthermore, natural killer (NK) associated receptor NKG2D was similarly expressed on aberrant IELs as on normal IELs (Table 2). Moreover, no differences in expression of the T-cell activation marker HLA-DR and IL15R-alpha were found on both cell types (Figure 2C-D; Table 2).

Analysis of cell cycle activity of the intestinal IELs using nuclear staining of proliferating cell nuclear antigen (PCNA) revealed a significantly lower MFI expression in aberrant IELs compared to the immunophenotypically normal IELs ($p<0.01$) (Figure 3G-I; Table 2). Even though only limited PCNA expression was observed in IELs, in all eleven patients tested PCNA levels were lower in the aberrant IEL population than in the normal IEL population. In contrast, the expression of the proliferation marker Ki-67 was similar on both cell types (Figure 3D-F; Table 2). Both markers were tested simultaneously but no correlation between the two was observed .

Table 2 Immunophenotype of aberrant IELs and normal IELs determined by flow cytometric analysis.

		Aberrant T-cells [median; range]		**Normal T-cells** [median; range]		**Mann Whitney** %	**Mann Whitney** MFI-index
	n	**%**	**MFI-index**	**%**	**MFI-index**	**p**	**P**
CD45RA	10	55.5 [20-99]	4.5 [1.4-17.4]	45.0 [20-97]	4.2 [1.8-15.9]	N.S.	N.S.
IL15R-alpha	14	3.2 [0.2-59.8]	1.0 [1-3.4]	9.6 [1.8-55.4]	1.3 [1-3.6]	,056	N.S.
Ki-67	15	8.8 [3.4-44.7]	1.9 [1.3-5.6]	6.2 [2.8-23.2]	2.1 [1.1-6.1]	N.S.	N.S.
PCNA	11	17.6 [2-70]	1.9 [1-3.8]	49.9 [7.5-75.8]	2.8 [2-4.7]	,047	,007
NKG2D	8	34 [5-67.2]	2.4 [1.3-3.4]	29.2 [15-74.8]	2.6 [1.4-3.2]	N.S.	N.S.
HLA-DR	8	34.5 [7.2-82]	5.8 [2.4-16.6]	53.3 [15.5-76.5]	7.7 [5.2-30]	N.S.	N.S.
Granzyme B	8	78.7 [48.7-96]	8.2 [3.8-92.4]	38.7 [29-69.3]	6.1 [2.1-16.5]	0.03	N.S.

Table 3 Rearrangement analysis of the TCR delta, gamma, and beta chains in small intestinal biopsies.

Patient	**TCR delta**	**TCR gamma**	**TCR beta**	**interpretation**
1	No amplification			
2	No clonality			
3	No clonality			
4	No clonality			
5	No clonality			
6	C	NC	NC	
7	C	C	NC	
8	C	C	NC	
9	NC	C	NC	V-J beta loss
10	NC	C	NC	V-J beta loss
11	NC	C	NC	V-J beta loss
12	C	NC	C	V-J beta loss; TCR beta incomplete
13	C	C	C	V-J beta loss; TCR beta incomplete
14	C	C	C	V-J beta loss; TCR beta incomplete
15	NC	C	C	V-J beta loss; TCR beta incomplete
16	NC	C	C	Complete TCR beta
17	NC	C	C	Complete TCR beta
18	C	C	C	Complete TCR beta

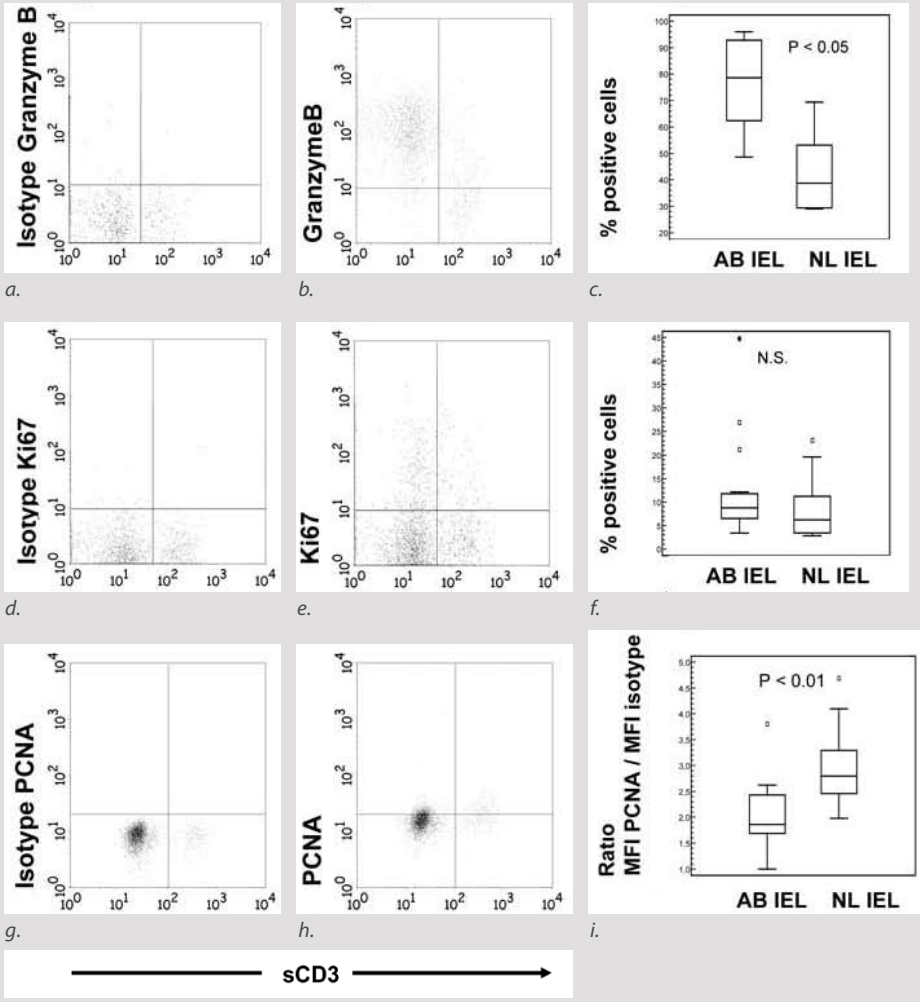
Abbreviations: **NC** no clonality; **C** monoclonal.

TCR rearrangement analysis

Detailed TCR rearrangement analysis was performed on duodenal biopsies of 19 well-defined RCD type 2 patients: 15 at time of diagnosis, 2 following cladribine therapy and 1 after cladribine with subsequent autologous stem cell transplantation. The patient characteristics are shown in table 1.

Aberrant IEL populations in RCD type 2 patients revealed a heterogeneous pattern of TCR rearrangements (Table 2). In one sample the DNA quality was probably inadequate (patient 1) as no amplification was found. The other cases could be subdivided into four groups. In the first group (patients 2-5) a polyclonal signal was detected. The second group (patients 6-8) revealed a monoclonal TCRD and/or TCRG gene rearrangement. The majority of the patients was present in the third group (patients 9-16) and all contained monoclonal TCRD and/or TCRG as well as incomplete TCRB gene rearrangements. The last group (patients 17-19) showed monoclonal TCRD and/or TCRG gene rearrangements, while a complete, monoclonal TCRB gene rearrangement was observed as well. Remarkably, all three patients in the last group, but none in the other groups, developed an EATL. No correlation between the above described groups and expression of any of the phenotypical markers was observed. Furthermore, in two patients (9 and 13) no clonal TCRG gene rearrangements were found, whereas the TCRB and TCRD gene rearrangements clearly revealed a monoclonal population.

In addition, the presence of TCR gene rearrangements in RCD cell line P1, P2 and P3 was assessed with multiplex PCR analysis and Southern blot analysis. PCR-based analysis indicated that in RCD cell line P1 TCRD gene rearrangements were absent while rearrangement of the TCRG gene and TCRB gene were, respectively, nonfunctional and incomplete (Table 4). Southern blot analysis of the TCRD gene- and TCRB gene rearrangements confirmed these data as bi-allelic loss of the TCRD gene and incomplete rearrangement of the TCRB gene were found (Figure 4). Bi-allelic loss of the TCRD gene could point to rearrangement of both TCR-alpha (TCRA) alleles in RCD cell line P1. RCD cell line P2 displayed incomplete TCRD gene rearrangement, nonfunctional TCRG gene rearrangement and no rearrangement of the TCRB gene (Table 4) Southern blot analysis indicated that one allele of the TCRD gene was lost while rearrangement of the other was incomplete (data not shown). Furthermore, the TCRB gene was in germline configuration indicating that no rearrangements of the TCRB gene were made in RCD cell line P2 (data not shown). In contrast to RCD cell line P1 and P2, no TCR gene rearrangements were found with multiplex PCR for RCD cell line P3 (Table 4) and with Southern blot analysis of this cell line only germ line configuration of the TCR genes was found (data not shown). These data indicate that RCD cell lines can display a diverse phenotype regarding the TCR gene rearrangements, characterized by incomplete, non-functional TCR gene rearrangements or even total absence of TCR gene rearrangements (Table 4).



*Intracellular expression of Granzyme B **(b)**, Ki67 **(e)** and PCNA **(h)** in aberrant IELs (Ab IEL) and normal IELs (NL IEL) derived from case 9, case 5 and case 13 (Table 1), with corresponding isotype controls **(a, d, g)** illustrate representative data from individual patients. Depending on distribution, expression of Granzyme B **(d)** and Ki67 **(f)** was reported as percentage positive cells, while MFI was used to report PCNA expression which was correlated to the MFI of the isotype control **(i)**. Aberrant IEL populations revealed significant ($p<0.05$) higher Granzyme B expression **(c)** and significantly lower PCNA expression ($p<0.01$) as compared to normal IELs **(i)**.*

Figure 3.

Table 4 Rearrangement analysis of the TCR delta, gamma, and beta chains in three RCD type 2 cell lines.

Cell line	TCR delta	TCR gamma	TCR beta	interpretation
P1	NC	C	C	TCR delta: D-J rearrangement TCR gamma: non-functional V
P2	C	C	NC	TCR-delta: incomplete; TCR gamma: 1 out of frame, 1 non-functional V
P3	No rearrangements			
$CD4^+$ TCR alpha-beta+	NC	C	C	Rearrangements fitting with TCR alpha-beta phenotype

DISCUSSION

Although it is generally accepted that EATL in RCD type 2 patients originates from a clonal expansion of aberrant T-lymphocytic population located in the small intestine that tends to disseminate to the blood and the entire gastrointestinal epithelium,[31] it is unclear for what reason this has been observed in only half of the cases. Therefore, this study aimed to gain insight in the immunophenotypic characteristics and origin of aberrant IELs.

In this study, flow cytometric analysis revealed an increased granzyme B expression on aberrant IELs over its normal counterpart, indicating a differentiation towards a more cytotoxic phenotype, possibly in consequence of a continuous pro-inflammatory environment. In uncomplicated CD normal $CD3^+$ IELs are thought to be reprogrammed into cytotoxic NK-like T-cells[32], and data is emerging that aberrant IELs also differentiated towards a cytotoxic NK-like phenotype with upregulation of KIRS, granzyme H and NKG2D.(Tjon et al. manuscript in preparation) Due to their cytotoxic phenotype aberrant IELs may be involved in the gluten-independent destruction of enterocytes in RCD type 2. However, since aberrant IELs lack a TCR, a TCR-independent mechanism is required in this process. Recently, it was shown that aberrant IELs, in a subset of RCD type 2 patients, can acquire the ability to lyse epithelial cells via DNAM-1.[33] Nevertheless, a TCR independent mechanism is known to be involved in uncomplicated CD as well, since NKG2D expressed by cytotoxic T lymphocytes is able to bind to its ligand MICA that is expressed on enterocytes, and subsequently induces apoptosis of these enterocytes.[34,35] Therefore, NKG2D expression on aberrant IEL was evaluated, which revealed a similar NKG2D expression on aberrant IEL compared to normal IELs.

IL-15 has an anti-apoptotic effect on aberrant IELs and by doing so it plays an important role in the expansion of the aberrant IEL population.[16] Given the observation that the IL-15 threshold for IEL expansion in CD patients appears to be linked to an increased expression of IL15R-alpha on these IELs, in combination with the report that a substantial number of EATL-cells expressed IL15R-alpha, we compared IL-15R-alpha expression on aberrant and normal IELs, but did not find any difference in expression.[36,37] It must be noted that although IL15R-alpha has the highest affinity for IL-15, recently it became clear that IL15R-alpha is not necessary for IL-15 induction since most IL-15 transduction is directed by the beta/gamma chain.[38]

In contrast to Malamut and colleagues who observed low Ki-67 expression in aberrant IELs,[16] our flow cytometric analysis showed a substantial percentage of the aberrant IELs expressing intracellular Ki-67, yet comparable to that in normal IELs. This suggests that aberrant IELs do proliferate to a similar extent as normal IELs, and together with the anti-apoptotic effect of IL-15 specifically on aberrant IELs, may provide the aberrant IEL population with a survival benefit with subsequent expansion in the intestinal mucosa. Furthermore, our results revealed a significant decrease of PCNA expression in aberrant IELs, with a concomitant non-different Ki-67 expression. Ki-67 and PCNA are both considered proliferation markers, whereas the latter has been implicated in the process of DNA-mismatch repair as well.[39] Therefore, the decreased PCNA expression found in aberrant IELs compared to its normal counterpart is unlikely the result of a lower proliferation and might rather indicate an impaired DNA-mismatch repair

system in these cells. These findings are in line with previously reported chromosomal damage in aberrant IELs, and may facilitate further transformation of aberrant IELs into EATL.[40] Our findings might be influenced by previous treatment with cladribine, that was administered to all but one patient studied. Nonetheless, we recently reported that cladribine induces only a limited reduction of the percentage of aberrant IEL in 40% of cases, while the majority still harbours a substantial aberrant IEL population after cladribine treatment.[29] This suggests that cladribine exerts a similar effect on aberrant and normal IELs, and therefore we feel that the comparison of the phenotype of both cell types provides useful information regarding their respective characteristics.

Currently it remains unclear whether this aberrant population originates from dedifferentiating mature IELs, or that it regards a monoclonal expansion of a unique, physiological subpopulation.[21,22] In this study, a heterogeneous TCR gene rearrangement pattern in duodenal biopsies of a relatively large series of RCD type 2 patients was observed, and confirmed in three RCD type 2 cell lines. Based on TCR gene rearrangements four groups could be distinguished. First, a small group of RCD type 2 patients showed no clonal TCR gene rearrangements indicating either a NK-cell origin, clonal T-cells in an early stage of their development prior to TCR-rearrangements, or simply a polyclonal T-cell population. The second group contained clonal TCRD and TCRG gene rearrangements, but no TCRB gene rearrangements, suggesting either a γδ T-cell origin or a deranged early developing T-cell. The latter results were in agreement with findings by Cerf-Bensussan and colleagues, who reported clonal TCRD/TCRG gene rearrangements in several RCD type 2 patients.[19] The fourth group displayed complete, monoclonal TCRB gene rearrangements. It must be noted however, that mature αβ T-cells not only contain a complete beta chain, but subsequently also rearrange an alpha chain. TCRA gene rearrangements were not determined in this study due to lack of RNA, so from these results it cannot be concluded that these monoclonal populations are derived from fully mature T-cells. Strikingly, all three patients in the fourth group developed an EATL in a later stage, possibly indicating that secondary EATL evolve from T-cells that have reached a certain stage of maturity. These findings are in agreement with the current WHO-classification which classifies EATL as a mature T-cell neoplasm.[41] It could be hypothesised that these more mature cell populations are less responsive to currently available therapies.

Furthermore, the phenotype of RCD cell line P1 is closest to that of a mature T-cell as analysis of TCR gene rearrangements suggest functional rearrangement of the TCRA gene but demonstrate incomplete rearrangement of the TCRB gene. This is also in line with the previous report describing that replacement of the TCRB chain restores TCR αβ expression in this cell line.[24] RCD cell line P2 could represent an immature T-cell as TCR rearrangements were initiated in this cell line. The absence of TCR rearrangements in RCD cell line P3 could indicate that this cell line represents an even earlier stage in T-cell- or NK cell development. Taken together, the TCR rearrangement analysis strongly suggest that the majority of the aberrant IEL populations in RCD type 2 patients originates from a monoclonal expansion of immature T-cells, that deranged in their development before reaching full maturity. The latter finding is supported by the lack of expression of the early T-cell development-associated markers TdT, CD1alpha and

CD34 by aberrant IELs. Furthermore, evolvement into an EATL was only observed in patients harbouring the most mature TCR rearrangement pattern. Although this patients series is small, this might be, at least in part, an explanation for the clinical observation that half of the RCD type 2 patients eventually develop an EATL, but further research addressing the cause of this abnormal development is warranted.

For daily clinical practise it is relevant that two RCD type 2 patients with a phenotypically aberrant IEL population revealed clonal TCRB or TCRD rearrangements however displayed polyclonal TCRG rearrangements. This can be explained by the fact that TCRG gene rearrangements are present in most, but not all αβ T-cells.[42] Moreover, these results confirmed our previous findings that clonality analysis of only the TCRG gene rearrangement, as often used in the workup of RCD, misses at risk monoclonal populations, and that phenotypical identification of an aberrant population is a superior predictor of EATL development.[7] Therefore, TCRB gene rearrangement analysis in addition to phenotypical identification of aberrant IEL could be useful to identify at risk aberrant IEL population. Further prospective studies are needed.

CONCLUSION

In conclusion, this study showed that aberrant IELs originate from deranged developing precursor T-lymphocytes and display clear differentiation to a cytotoxic phenotype. Aberrant IEL populations between RCD type 2 patients displayed different stages of maturity, of which only the patients harbouring the most mature aberrant IEL populations developed an EATL.

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Chapter 9

Differential IL-13 production by small intestinal leukocytes in active coeliac disease versus refractory coeliac disease

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ABSTRACT

A small fraction of coeliac disease (CD) patients have persistent villous atrophy despite strict adherence to a gluten-free diet. Some of these refractory CD (RCD) patients develop a clonal expansion of lymphocytes with an aberrant phenotype, referred to as RCD type 2. Pathogenesis of active CD (ACD) has been shown to be related to gluten-specific immunity whereas the disease is no longer gluten driven in RCD. We therefore hypothesized that the immune response is differentially regulated by cytokines in ACD versus RCD type 2 and investigated mucosal cytokine release after polyclonal stimulation of isolated mucosal lymphocytes. Secretion of the TH2 cytokine IL-13 was significantly higher in lamina propria leukocytes (LPLs) isolated from RCD type 2 patients as compared to LPL from ACD patients ($P = 0.05$). In patients successfully treated with a gluten-free diet LPL-derived IL-13 production was also higher as compared to ACD patients ($P = 0.02$). IL-13 secretion correlated with other TH2 as well as TH1 cytokines but not with IL-10 secretion. Overall, the cytokine production pattern of LPL in RCD type 2 showed more similarities with LPL isolated from GFD patients than from ACD patients. Our data suggest that different immunological processes are involved in RCD type 2 and ACD with a potential role for IL-13.

INTRODUCTION

Coeliac disease (CD) is an autoimmune enteropathy that is triggered by the gliadin fraction of dietary gluten peptides.[1] The immune processes in CD have been widely studied and it is commonly accepted that in CD innate and adaptive immune responses are part of the pathogenesis.[2] Gliadins can exert direct toxic effects by binding to epithelial cells, resulting in the production of IL-15 and TNFα.[3-5] IL- 15 upregulates natural-killer receptors on intraepithelial cytotoxic T lymphocytes as well as their ligands on epithelial cells, which leads to enhanced apoptotic killing of epithelial cells.[6] The main pathogenic mechanism of CD, however, is believed to be a gluten-specific TH1-mediated response resulting in an overexpression of IFNγ in the (intra) epithelial compartment.[7] IFNγ, together with TNFα, enhances the expression of transglutaminase-2 (TG2).[8] TG2 binds and deamidates gliadin peptides, which leads to a better presentation of gliadin peptides to specific TH cells and a subsequent stronger gliadin-specific immune response with even higher amounts of IFNγ.[9,10] Although the exact mechanism is unknown, evidence exists that the overexpressed IFNγ ultimately leads to the mucosal damage found in CD.[11,12] More recently, the proinflammatory cytokine IL-17A has been found to play an important role in coeliac pathology as well.[13] Despite a predominant proinflammatory cytokine profile in active CD, also expression of the regulatory cytokine IL-10 is found, possibly limiting the production of proinflammatory cytokines.[14] Indeed, in a pilot phase I study, treatment with recombinant IL-10 did induce some relief of symptoms in a minority of patients but IL-10 treatment did not lead to mucosal recovery.[15] In contrast to uncomplicated CD, less is known about the pathology of refractory coeliac disease (RCD).[16] RCD is a complication of CD in which patients despite following a strict gluten-free diet (GFD) do not recover from symptoms and mucosal lesions. RCD type 2 is characterized by a significant (>20%) aberrant intraepithelial T-lymphocyte (IEL) population in the small intestinal mucosa. These aberrant IEL lack T-cell-specific surface markers, that is, T-cell receptor (TCR), CD3, CD4, and CD8, but express cytoplasmic CD3. Clonal expansion of these aberrant IEL is thought to be responsible for the occurrence of enteropathy-associated T-cell lymphoma (EATL), which occurs in 60%– 80% of RCD type 2 patients within 5 years.[17] Similarly to uncomplicated, active CD, IL-15 and IFNγ are reported to be enhanced in RCD; however it is unclear whether they play significant roles in the pathogenesis of RCD.[18,19] TNFα may play a role in RCD, since some RCD cases have been described where anti-TNFα therapy has shown to have a beneficial effect.[20,21] IL-17A, IL-13, and IL-5 have not yet been investigated in RCD. As in RCD the immunological trigger gliadin is absent, we hypothesized that the cytokine profile of IEL and lamina propria leukocytes (LPL) is altered as compared to the gliadin driven immune response in ACD. Therefore, we measured protein levels of the proinflammatory cytokines TNFα, IFNγ, and IL-17A, the TH2 cytokines IL-13 and IL-5, and the regulatory cytokine IL-10, in supernatants of polyclonally stimulated leucocytes from biopsies of uncomplicated CD and RCD patients.

PATIENTS AND METHODS

Patients

Consecutive patients (n = 20) were included in our study that visited our outpatient clinic for CD or RCD follow-up. Biopsies were taken for diagnostic purposes and cells remaining from the diagnostic procedure were used for our experiments. The study protocol adhered to the guidelines set by our institutional ethical committee. Patients with concomitant complications such as ulcerative jejunitis or autoimmune enteropathy and patients with collagenous sprue were excluded. Active CD (ACD) was diagnosed according to current guidelines for adult CD,[22] that is, if biopsies showed increased numbers of intraepithelial lymphocytes, crypt hyperplasia, and villous atrophy together with antibodies against transglutaminase-2 (TGA) and endomysium. CD patients were prescribed a gluten-free diet (GFD) and were considered recovered when TGA levels normalized and when follow-up biopsies showed no villous atrophy anymore (Marsh 0–II; GFD patient group). Adherence to a GFD was confirmed by a dietitian and absence of TGA in serum. Follow-up biopsies were taken in order to confirm histological recovery or when CD symptoms persisted and RCD was suspected. Patients were diagnosed with RCD when malabsorption symptoms and histological abnormalities persisted or recurred despite strict dietary adherence (as confirmed by the disappearance of TGA and EMA) and after exclusion of other intestinal diseases. RCD type 2 was diagnosed, if an aberrant IEL population ($CD3^-$, intracellular $CD3^+$, $CD7^+$) occurred with a frequency of more than 20% of all IEL.[23] Since the distinction between RCD type 1 and slow responders on a GFD can only be done after a long-term follow-up, patients with suspected RCD type 1 were excluded and only patients with RCD type 2 were included in this study. RCD type 2 patients were treated with autologous stem cell transplantation (SCT), 6-thiogunidine (6-TG), cladribine, or entocort; one patient was analyzed prior to treatment (Table 2). Similarly to CD patients, RCD type 2 patients were considered recovered, when villous atrophy was absent after therapy.

Cell Cultures and Cytokine Measurement

Small intestinal biopsies were separated into epithelial layer and lamina propria by incubation in PBS containing DDT and EDTA in a 37°C shaking water bath for one hour as previously described.[24] IEL were washed and collected in ice-cold PBS-BSA 0.1%.The remaining lamina propria was incubated for 2 h in PBS with 10% FCS and 0.16 U/mL collagenase (Collagenase A, Roche). After incubation the biopsies were passed through a sterile 100 μm and filtered through a sterile 40 μm mesh. Cells were then washed and collected in ice-cold PBS containing 0.1% BSA. IEL and LPL were incubated for at least 15 min. with magnetic beads linked to anti-CD45 antibodies (MACS human-CD45 MicroBeads, Miltenyi Biotec). $CD45^+$ cells (leukocytes) were separated on a magnetic column (MACS MS column, Miltenyi Biotec), collected, and divided over two (IEL) or three (LPL) wells of a 96-well cell-culture plate: IEL: (1) unstimulated, (2) stimulated with 50 ng/mL PMA, 1 μg/mL ionomycin, and 50 ng/mL LPS; LPL: (1) unstimulated, (2) stimulated with 50 ng/mL PMA and 1 μg/mL ionomycin, and (3) stimulated with 50 ng/mL LPS. Each well contained the cells of approximately 2 biopsies in a total volume of 100 μL. After

24 hour incubation at 37°C and 5% CO2, supernatants were collected, frozen, and stored at –20°C until analysed. Cytokine levels of TNFα, IL-17A, IL-13, IL-10, and IL-5 were determined using amultiplex bead assay (Cytometric Bead Assay, BD). IFNγ was measured using a commercially available ELISA kit (PeliKine compact human IFNγ, Sanguin).

FACS Analyses

Cell subsets, that is, $CD4^+$ and $CD8^+$ T-cells, $CD3^-$ $CD16/56^+$ NK cells, and $CD19^+$ B cells, were determined by multicolour FACS analysis using CD3- FITC, CD8-PE, CD45-PerCP, and CD4-APC and CD3- FITC, CD16/56-PE CD45-PerCP, and CD19-APC antibody conjugates, respectively (Multitest, BD). Aberrant IEL were analysed by surface CD3, CD52, and CD45 followed by cytoplasmic staining of CD3 after cell permeabilization (Cytofix/CytoPerm Plus kit, BD Biosciences). All analyses were performed on lymphocytes, basedonbrightCD45 staining and low side scatter (SSC). Aberrant T-cells were defined as $CD52^+$ cytoplasmic $CD3^+$ and surface$CD3^-$ cells. Total numbers of IEL (cell harvest) were determined using FACS tubes containing a fixed number of reference beads (Trucount tubes, BD).

Statistical Analyses

Differences in cytokine levels were tested with the Mann-Witney U test. Difference in sex distribution was tested with the chi-square test. Differences in age, cell count, and cell type ratios were tested with the student's t-test. Correlation coefficients were calculated with a two-sided Pearson's correlation.

RESULTS

Patient Characteristics and Composition of Leukocyte Infiltrates

A total of 20 patients were included in our study: 4 patients with active coeliac disease (ACD), 7 on a gluten-free diet (GFD), and 9 patients with RCD type 2. RCD type 2 patients tended to be older at the time of cytokine analysis than ACD patients ($P = 0.07$, Table 1). The follow-up time of GFD patients was at least 8 months, and that of RCD type 2 patients at least 2 years since the start of the gluten-free diet (data not shown). Five of the RCD type 2 patients had villous atrophy. One of these patients was not treated and four retained villous atrophy despite treatment (Table 2). Of the four patients that recovered histologically after treatment, one was treated with SCT, and the other three with cladribine. Cell yield (total number of isolated IEL) did not differ significantly between groups. The median cell yield was highest in ACD patients with 28,500 cells per biopsy compared to GFD (16,000 cells per biopsy) and RCD TYPE 2 (19,100 cells per biopsy). Due to large variation, however, no significant difference in cell yield was observed between groups. The percentage of $CD3^+$ IELs, mostly $CD8^+$ T-cells, was significantly lower in RCD type 2 patients compared to GFD and ACD, which is due to the high percentage of aberrant IEL found in RCD type 2 patients (Table 1). NK cell frequencies in IEL were generally low (Table 1) and B cells were absent (data not shown). In the LPL fraction NK cell and B cell frequencies were below 10% in all groups (Table 1).

Table 1.

	ACD (n=4)	GFD (n=7)	RCD type 2 (n=9)
Sex, females	75.0%	71.4%	33.3%
Age, yrs	45.8 (22.2–75.3)	55.9 (35.3-72.0)	70.6(41.7-76.2)
Villous atrophy, %	100%	0.0%	45.5%
Cell yield, 10^3 IEL/biopsy	28.5 (7.5-58.0)	16.0 (2.1-113.0)	19.1 (5.8-62.4)
$CD3^+$ IEL, % of CD45	99 (97-99)	96 (86-98)	21 (10-99)
$CD4^+$ IEL, % of CD45	5 (1-10)	3 (2-32)	5 (1-14)
$CD8^+$ IEL, % of CD45	77 (59-86)	78 (65-90)	13 (5-69)*
$CD16/56^+$ IEL, % of CD45	1 (0-3)	2 (1-11)	3 (0-24)
aberrant IEL, % of CD45	0 (0-1)	2 (0-6)	66 (1-87)*
$CD3^+$ LPL, % of CD45	40-43	21-60	25-38
$CD4^+$ LPL, % of CD45	13-28	0-31	8-24
$CD8^+$ LPL, % of CD45	10-16	2-29	4-14
$CD16/56^+$ LPL, % of CD45	3-3	5-8	1-4
$CD19^+$ LPL, % of CD45	4-5	1-8	3-10

For age and IEL data medians (5 percentile-95 percentile) are shown. For LPL data ranges are shown, since data for composition of LPL was available only in 2 ACD patients, 4 GFD patients, and 4 RCD type 2 patients. *Significantly lower percentage of $CD3^+$ and $CD8^+$ cells compared to ACD and GFD (due to high percentage of aberrant T-cells).

Cytokine Levels in IEL

Stimulation of IEL overall resulted in low cytokine levels, probably due to the generally low numbers of leukocytes present in the epithelial layer. Only IFNγ and TNFα, both known to be increased in the duodenum of CD patients, reached detectable levels in IEL. In order to analyse whether IEL numbers may influence possible differences in cytokine levels between the groups, the amount of cytokine was divided by the number of IEL that were isolated from biopsies. No significant differences could be found between ACD and RCD type 2 patients whether the amount of cytokine per 1000 IEL (Figures 1(a) and 1(b)) or the amount of cytokine per two biopsies (Figures 1(c) and 1(d)) was analyzed. IFNγ production was not lower in GFD patients as compared to ACD patients. However, in the RCD type 2 group, IEL-derived IFNγ production was the highest in patients with persisting villous atrophy (Figures 1(a) and 1(b), closed symbols).

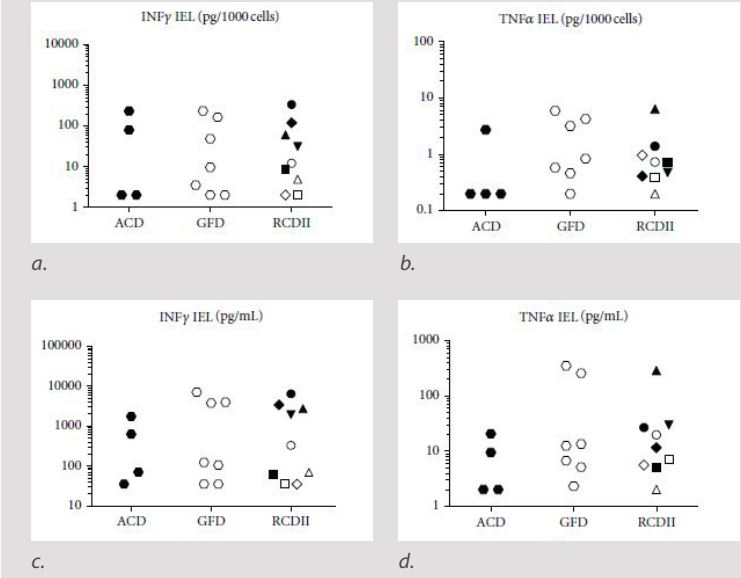


Figure 1.

*Production of INFγ and TNFα by IEL from active CD patients (ACD), patients on a gluten-free diet (GFD), and refractory CD type 2 (RCDII) patients after PMA/ionomycin/LPS stimulation. RCD type 2 patients with villous atrophy (closed symbols); RCD type 2 patients without villous atrophy (open symbols); for individual characteristics see Table 2. ((**a), (c)**) IFNγ and ((**b), (d)**) TNFα production. ((**a), (b)**) Production per 1000 IEL or ((**c), (d)**) per Ml per two biopsies.*

Cytokine Levels in LPL

LPLs were stimulated with either PMA/ionomycin to trigger all the leukocytes or LPS to trigger antigen-presenting cells (APC) only. After LPS stimulation most cytokines were undetectable and only low levels of IFNγ and TNFα were detectable in a minority of the patients. For both IFNγ and TNFα no differences could be observed between groups after LPS stimulation (data not shown). As in general the levels were 50- to 500- fold lower after LPS stimulation

as compared to PMA/ionomycin stimulation, IFNγ and TNFα production after PMA/ionomycin will be mostly lymphocyte rather than APC derived. In contrast to LPS, stimulation of LPL with PMA/ionomycin resulted in detectable cytokine levels. RCD type 2 patients who were treated within 6 weeks before the biopsy was taken appeared not to be different in terms of cytokine production from patients who were treated more than 6 weeks before the biopsy was taken (Figure 2 and Table 2).

Table 2.

	Sex	Age, yrs	Marsh	Treatment	Last treatment<6 weeks before biopsy	Aberrant cells, % of CD45	Symbol Figures 1 and 2[d]
1[a]	M	68.1	IIIa	Chemotherapy, entocort	Yes	77%	●
2[c]	M	76.0	IIIa	2x cladribine	Yes	37%	■
3	F	72.8	IIIa	Cladribine	No	70%	▲
4	F	41.7	IIIb	None	No	87%	▼
5[b]	F	54.9	IIIc	6-TG	Yes	0.6%	◆
6	M	70.3	I	Cladribine, SCT	No	13%	○
7[c]	M	76.2	0	Cladribine	Yes	41%	□
8	M	72.9	I	SCT	No	73%	△
9	M	70.6	I	Cladriine	No	66%	◊

[a] ***RCD type 2 after successful treatment of enteropathy-associated T-cell lymphoma***
[b] ***Enteropathy-associated T-cell lymphoma was diagnosed when biopsy was taken***
[c] ***Patients 2 and 7 are the same patients before and after histological recovery***
[d] ***Corresponding symbol in Figures 1 and 2***

However, levels of most cytokines (IFNγ, TNFα, IL-13, and IL-17A) tended to be the highest in patients with persisting villous atrophy (Figure 2, closed symbols). Similar to the IEL results, IFNγ production by LPL was comparable between ACD and RCD type 2 patients and IFNγ production was not reduced in GFD patients compared to ACD (Figure 2(a)). IL-13 responses were higher in RCD type 2 when compared to ACD patients but were also higher in GFD as compared to ACD (Figure 2(c)). Since IL-13 production was significantly increased in RCD type 2 patients as compared to ACD patients, we analyzed the coexpression of IL-13 and the other cytokines by calculating correlation coefficients for all IL-13 cytokine pairs. IL-13 release correlated the strongest with IL-17A and TNF ($r = 0.80$ and $r = 0.73$, resp.; both $P < 0.001$; Figures 3(a) and 3(b)). Weaker correlations were observed with IL-5 and IFNγ ($r = 0.63$, $P = 0.003$ and $r = 0.45$, $P = 0.04$, resp.; Figures 3(c) and 3(d)), while there was no significant correlation between IL-13 and IL-10 ($r = 0.38$, $P = 0.10$; Figure 3(e)).

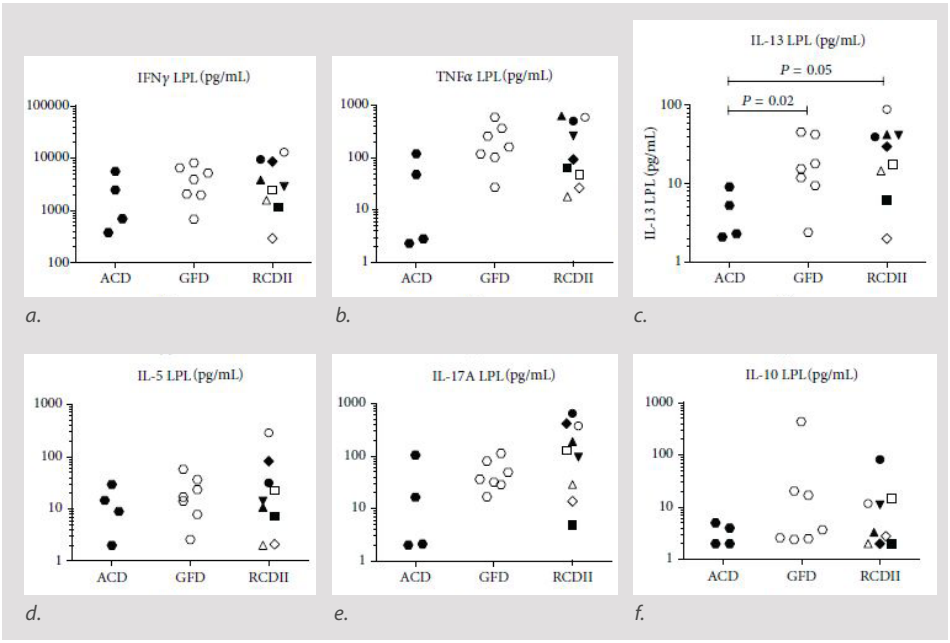
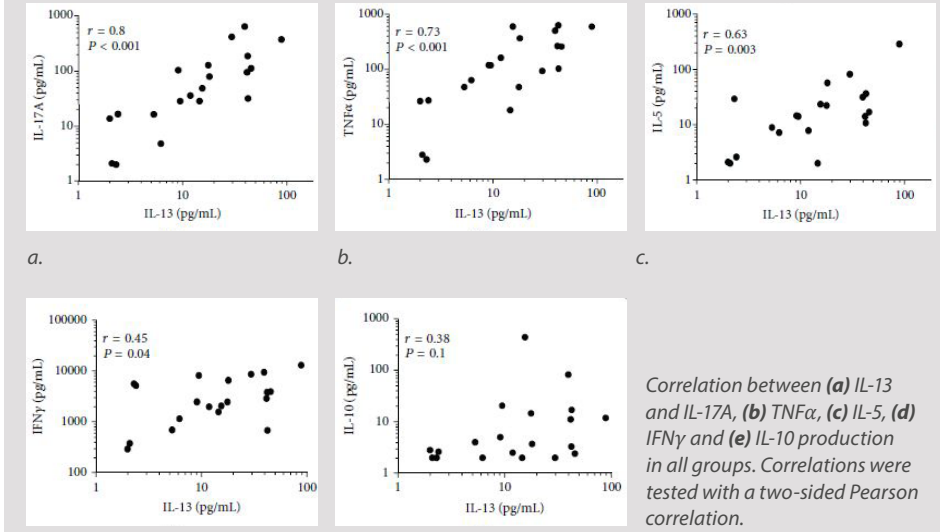


Figure 2.

*Production of **(a)** IFNγ, **(b)** TNFα, **(c)** IL-13, **(d)** IL-5, **(e)** IL-17, and **(f)** IL-10 by LPL from active CD patients (ACD), patients on a gluten-free diet (GFD), and refractory CD type 2 (RCDII) patients after PMA/ionomycin stimulation. RCD type 2 patients with villous atrophy (closed symbols); RCD type 2 patients without villous atrophy (open symbols). Groups were compared using the Mann-Whitney U test. P values are shown for significant differences.*



*Correlation between **(a)** IL-13 and IL-17A, **(b)** TNFα, **(c)** IL-5, **(d)** IFNγ and **(e)** IL-10 production in all groups. Correlations were tested with a two-sided Pearson correlation.*

Figure 3.

DISCUSSION

In this study we tested the hypothesis that the local cytokine profile would be different in gluten-driven ACD as compared to gluten-independent RCD type 2. This was investigated by analyzing the capacity of LPL and IEL isolated from the duodenum of ACD and RCD type 2 patients as well as from patients successfully treated with a gluten-free diet to produce IFNγ, TNFα, IL-17A, IL-13, IL-5, and IL-10. IL-15 was not analyzed as it is not well secreted and unstable.[25] IFNγ production has been extensively studied in ACD and GFD. While IFNγ has been considered to play an important role in enterocyte destruction in ACD, several studies have shown that IFNγ levels are not reduced in GFD.[26,27] This is in line with our findings that show no difference in the capacity to produce IFNγ between IEL/LPL from ACD and GFD patients. Here, we also show that there is no increase in IFNγ production in RCD type 2 patients. This suggests that the capacity of IEL/LPL to produce IFNγ appears not to be solely dependent on an ongoing gluten-driven immune response. In contrast to our findings here, levels of TNFα protein have been found to be elevated in lamina propria and epithelium of ACD patients and decreased after a GFD.[28,29] However, there are important methodological differences between the present and these previous studies. While we used PMA and ionomycin stimulation to analyze the capacity of the IEL/LPL to produce particular cytokines, the above mentioned studies used RT-PCR analysis or immunohistochemistry to analyze cytokine mRNA levels or protein without prior stimulation. This suggests that the capacity of IEL and LPL to produce TNFα may be similar in ACD and GFD while the current production at the time of biopsy may be reduced in GFD. Although there is a considerable overlap between the groups, the capacity of LPL to produce IL-13 and IL-17A seems to be lower in ACD as compared to RCD type 2 and GFD, which reached statistical significance for IL-13 when analyzed individually. In pediatric ACD patients, lower numbers of mucosal T-cells with the capacity to produce IL-17A were observed as compared to controls. It was suggested that the relative lack of IL-17A producing T-cells may affect the homeostasis of the epithelial layer and contribute to increased intestinal permeability.[30] In our dataset this was less apparent; however, in a subset of RCD type 2 patients (particularly those with persistent villous atrophy despite treatment) high levels of IL-17A were detected after polyclonal stimulation and in only one of the ACD patients, suggesting a differentially driven IL-17A response in treatment-resistant RCD type 2 patients. This increased capacity of LPL to produce IL-17A in treatment-resistant RCD type 2 may be related to the continued inflammation and risk of EATL development, as IL-17A is involved in chronic inflammation as well as in tumor formation.[31]

To the best of our knowledge this is the first study that investigated local IL-13 levels in CD and RCD. In our experiments we found higher IL-13 production in RCD TYPE 2 patients as compared to ACD patients. IL-13 production capacity was also higher in GFD patients compared to ACD. Although IL-13 is mainly associated with airway pathology, it also has an important role in gut defense and inflammation.[32] In ulcerative colitis the high levels of IL-13 are shown to be derived from variant CD1d-restricted NKT-cells and IL-13 has been shown to have a toxic effect on colonic epithelial cells.[33,34] IL-13 has also been shown to be produced by NK cells as part of an innate response.[35] This is in line with the high levels of IL-13 found in RCD type 2 where

antigenic stimulation by gluten is lacking. The higher IL-13 production was not related to NK cell frequencies; whether the IL-13 produced is NK or variant NKT-cell derived remains to be investigated. IL-13 production capacity was not only correlated to IL-17A production but also to the other TH1 and TH2 cytokines, but not to the regulatory cytokine IL-10, which is in line with a proinflammatory role for this cytokine. IL-13 has been shown to have direct cytotoxic effects on epithelial cells. It is, therefore, intriguing to speculate why there is an increased capacity of LPL to produce IL-13 in both patients on a successful GFD and RCD type 2 patients. Differential expression of the receptors on epithelial cells as has been described for the IL-15 receptor[36] as well as regulatory cytokines not measured here (TGFβ) or contact-dependent regulation by regulatory cells may play a role. Although the difference between RCD type 2 and ACD was only statistically significant for the IL-13 production capacity, the production pattern of the other cytokines was comparable, and the overall cytokine profile of LPL in RCD type 2 showed more similarities with LPL from GFD patients than from ACD patients. It has to be taken into account that for this study we did not have healthy controls available to compare our results to. It is therefore unclear whether GFD patients and RCD patients had increased IL-13 levels or ACD had reduced IL- 13 levels.

CONCLUSION

In conclusion our data show that IL-13 production is lower in the lamina propria of ACD patients, compared to GFD and in particular RCD type 2 patients, suggesting that the immune responses in ACD and RCD type 2 are differently regulated and that IL-13 may play a role as a proinflammatory cytokine in the pathogenesis of RCD type 2.

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Chapter 10

Genetic variations in interleukin 12 related genes in autoimmune disease

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ABSTRACT

The interleukin-12 (IL-12) family comprises a group of heterodimeric cytokines and their respective receptors that play key roles in immune responses. A growing number of autoimmune diseases has been found to be associated with genetic variation in these genes. Based on their respective associations with the IL-12 genes, autoimmune diseases appear to cluster in two groups that either show strong associations with the Th1/Th17 pathway (as indicated by genetic association with IL12B and IL23R) or the Th1/IL-35 pathway as the consequence of their association with polymorphisms in the IL12A gene region. The genetic associations are described in relation to what is known of the functionality of these genes in the various diseases. Comparing association data for gene families in different diseases may lead to better insight in the function of the genes in the onset and course of the disease.

INTRODUCTION

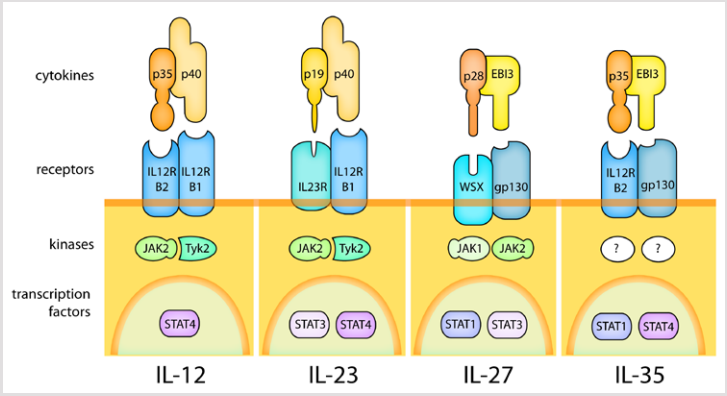
Autoimmune diseases (AID) are believed to occur as the consequence of a disbalance in the complex interplay between genetic and environmental factors. In the last decade significant progress has been made in the identification of genetic risk factors that underlie these diseases. One recurrent observation is that the known clinical overlap between these diseases has found its counterpart in a considerable genetic overlap.[1] The major challenge lies now in the interpretation of the vast amount of data that have been generated in Genome Wide Association studies (GWAS) and to link these data to the biology of the disease.

A growing number of AID has been found to be associated with genetic variation in the interleukin-12 (IL-12) family of genes. IL-12 related cytokine pathways play a pivotal role in T-cell activation and as such these polymorphisms may provide important clues to the mechanisms underlying autoimmune disease.

In the current review we aim to gain insight in the complex role of IL-12 in various immune-mediated diseases. Rather than reviewing the different genetic risk factors associated with a particular disease we here review the different diseases associated with a specific gene cluster. Based on their respective associations with specific IL-12 family members, we demonstrate that AID cluster in two groups. This finding is remarkable since AID clustering within these groups mirror to a certain extent the known clinical relationship between these diseases. Thus, for example, patients with inflammatory bowel disease (IBD) have an increased risk to develop psoriasis, sacroiilitis and spondylitis. As will be discussed here, these diseases show overlapping associations with gene regions within the IL-12/IL-23 gene cluster.

STRUCTURE AND FUNCTION OF THE IL-12 CYTOKINE FAMILY

The IL-12 cytokine family currently consists of 4 heterodimeric cytokines, interleukin-12 (IL-12), interleukin-23 (IL-23), interleukin-27 (IL-27) and interleukin-35 (IL-35) (Figure 1).



Structure of the IL-12 cytokine family, showing the combination of dimeric molecules that make up the cytokines and their receptors. In addition the kinases and known transcription factors that are crucial for the intracellular signalling are depicted.

Figure 1 IL-12 cytokine family.

IL-12 is a heterodimer formed by IL-12p35 (encoded by *IL12A*) and IL-12p40 (encoded by *IL12B*). IL-12 is produced by antigen-presenting cells (APC), phagocytic cells and B-cells in response to infection and favors naïve $CD4^+$ T-cells to develop into pro-inflammatory T-helper type 1 (Th1) cells, which secrete the pro-inflammatory cytokine interferon-γ (IFN- γ) (Figure 2). In addition, it enhances the generation of cytotoxic T lymphocytes and NK cells along with the augmentation of the cytotoxic activity of these cells and concomitantly suppresses differentiation of T-helper type 2 (Th2) cells.[2]

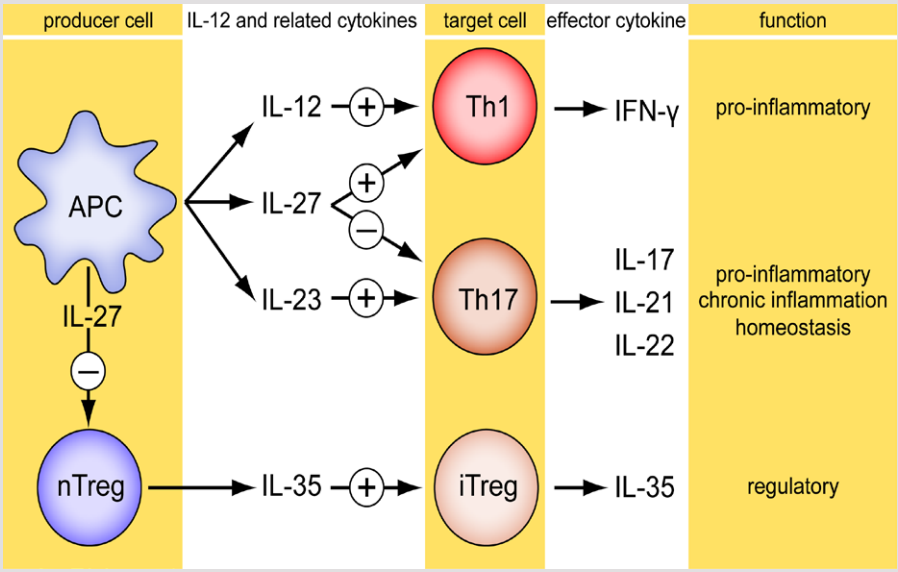
In addition to binding to the p35 subunit, IL-12p40 can form the pro-inflammatory cytokine IL-23 when bound to the IL-23p19 chain (encoded by *IL23A*). IL-23 has an important role in amplifying and stabilizing T-helper cells type 17 (Th17 cells), another pro-inflammatory cell population that is involved in combatting a wide range of infections,[3] and also plays an important role in autoimmune disease.[4] Th17 cells are characterized by secretion of IL-17A, IL-17F, IL-21 and IL-22 (Figure 2). Since IL-12 and IL-23 share the IL-12p40 subunit and IL-23 was more recently identified, many activities originally ascribed to IL-12 may in fact have been mediated by IL-23.

A more recently discovered cytokine, IL-27, is formed by Epstein-Barr-Virus induced molecule 3 (EBI3) and IL-27p28. Both proinflammatory (Th1), as well as anti-inflammatory functions (suppression of Th17 cell differentiation) have been ascribed to IL-27 (Figure 2).[5]

The latest member of the IL-12 family is IL-35, which is composed of IL-12p35 and EBI3. In contrast to the proinflammatory IL-12 family members IL-12 and IL-23, IL-35 is thought to have a role in controlling the immune response during active inflammation. In humans, IL-35 has been shown to suppress the proliferation of conventional T-cells as well as the conversion of

conventional T-cells into T regulatory cells that are named 'iTr35 cells' (Figure 2).[6,7]

A comparable mixing and matching of heterodimeric molecules is seen for the receptors for IL-12 and IL-23 which share the interleukin-12-receptor- β1 (IL-12Rβ1) chain together with a unique interleukin-12-receptor-β2 (IL-12Rβ2) and IL-23 receptor (IL-23R) chain, respectively (Figure 1). The receptor for IL-27 consists of a combination of WSX-1 (IL-27R) and gp130, the latter being a component of several cytokine receptors. The receptors for IL-35 were recently discovered to be composed of the IL-12 family related proteins IL-12Rβ2 and gp130.[8]



Antigen presenting cells (APC) are able to produce the majority of IL-12 related cytokines, IL-12, IL23, and IL-27. All of these affect the differentiation of naïve CD4 T-cells into T helper cells of either Th1 or Th17 type. The subsequent production of effector cytokines by the Th1 and Th17 cells leads to profound effects on inflammation and immunity. IL-35 is produced by the naturally occurring Tregs (nTreg) and can lead to the induction of peripheral Tregs (iTreg). They in turn also produce IL-35.

Figure 2 A schematic view of the members of the IL-12 related cytokines.

GENETIC ASSOCIATIONS OF AUTOIMMUNE DISEASE WITH THE IL-12 GENE FAMILY

When we analyzed studies reporting an association of AID with single nucleotide polymorphisms (SNPs) in any of the gene regions of the IL-12 cytokine family, it became clear that a surprising number of AID are associated with genetic variation in this gene family. This was especially apparent for *IL12A* and *IL12B*, and for the receptor chains *IL23R*, *IL12Rβ1* and *IL12Rβ2*. In Figure 3 the positions of the SNPs in these genes and the associations with the different diseases are depicted. For every association the current level of evidence was determined based on the size of the cohorts in which the association was observed as well as replication of the specific association in other study populations. In Table 1 these data are summarized, clearly showing that, based on the association of autoimmune disease with the various IL-12 regions, two major clusters can be distinguished. The first one encompasses Crohn's disease (CrD), ulcerative colitis (UC), psoriasis (Ps), psoriatic arthritis (PsA), ankylosing spondylitis (AS), and rheumatoid arthritis (RA). These diseases all show clear associations with the *IL23R*, and the majority is associated with the *IL12B* gene region as well. This indicates an important pathogenic role for the Th17, and possibly the Th1 pathway (Figure 1, 2). The second cluster includes primary biliary cirrhoses (PBC), coeliac disease (CD), multiple sclerosis (MS) and Graves disease (GD), which are all associated with genetic variations in the *IL12A* gene region, suggesting an important pathogenic role for IL-12 and/or IL-35 (Figure 1, 2). Finally, in some AID such as Type 1 diabetes (T1D), asthma and atopic dermatitis (AtD) associations with IL-12 gene region variants have also been reported but not yet widely replicated.

In the next section we will briefly summarize what is known about the involvement of these cytokines and their respective genetic associations in the various autoimmune diseases.

Table 1.

Cluster	Autoimmune disease	IL23R	IL12B	IL23A	IL12RB1	IL12A	IL12RB2	References
Th17 / Th1	Crohn's disease	++++	++++	-	-	-	-	13,14
	Ulcerative colitis	++++	++++	-	-	-	-	22
	Psoriasis	++++	++++	-	-	-	+	32,34
	Ankylosing spondylitis	+++	+++	-	-	-	_	41,42,44,45,47-49
	Rheumatoid arthritis	+++	-	-	-	-	_	53,54
Th1 / IL-35	Primary biliary cirrhosis	-	-	-	-	+++	+++	59-61
	Celiac disease	++	-	-	-	++++	-	85,89
	Multiple sclerosis	+	++++	-	-	++++	-	71,76,77
	Graves disease	-	-	-	-	++	-	82
Undefined	Asthma	-	++	-	-	+	-	87,91
	Atopic dermatitis	-	+	-	+	-	+	28,97
	Type 1 diabetes	-	+	-	-	-	-	102-104

The level of evidence for an association between AID and IL-12 related cytokine gene regions was based on the size of the cohort in which the reported association was found, as well as the size of other study populations in which a particular association was confirmed.

++++ ***Association found in large population (> 5000 patients)***
+++ ***Association found and replicated in medium size population (500 < patients < 5000)***
++ ***Association found but not replicated in medium size population (500 < patients < 5000)***
\+ ***Association found and possibly replicated in relatively small population(s) (< 500 patients)***
\- ***Association not found or not determined***

THE FIRST CLUSTER: ASSOCIATION WITH TH17 AND TH1

Inflammatory bowel disease

Ulcerative colitis (UC) and Crohn's disease (CrD) are chronic inflammatory diseases of the intestine, and are commonly referred to as inflammatory bowel disease (IBD). Immunologically, both diseases are distinct in that CrD is considered a Th1-mediated disease due to increased mucosal IL-12 and IFN-γ expression, while in UC a Th2 type response is dominant characterized by mucosal IL-13 expression.[9] More recent evidence suggests the involvement of IL-23 induced Th17 cells in the immune response in both types of IBD.[10]

Of all the autoimmune diseases that have been found to be linked to the IL-12 family, the genetics of IBD have been studied most extensively, as is illustrated by a recent meta-analysis that included over 22.000 CrD patients.[11] From these studies in various populations it has become apparent that CrD is associated with multiple polymorphisms in the *IL23R* gene region, that have been replicated in multiple populations (Table 1).[11-14] These associations include a SNP located in the ninth *IL23R* gene exon, a polymorphism situated in the 3'UTR, as well as various SNPs positioned in introns (Figure 3A).[11-14] Interestingly, these various polymorphisms in the *IL23R* gene exert varying risk effects, with some increasing disease susceptibility (intron 5) and others (exon 9) being protective. In addition to the association with *IL23R*, CrD has also been associated with polymorphisms located proximal of the *IL12B* gene (Figure 3B).[11,12,14]

Furthermore, CrD has been associated with genetic variants in *STAT3* (involved in Th17 downstream signalling), *STAT4* and *JAK2* (involved in Th1 and Th17 downstream signalling), as well *CCR6* and *TNFSF15* (involved in Th17 differentiation).[12,15] Extensive pathway analysis conducted in CrD identified the IL-12/IL-23 pathway, out of 534 pathways, as the strongest CrD associated pathway[16] lending further support for the role of Th17 cells in this disease. Regarding other members of the IL-12 gene family, an association with early-onset IBD and a polymorphism in proximity of the *IL27* gene (+320kb) was reported,[17,18], which correlated with decreased colonic mRNA IL-27p28 expression.[18] These findings contradict an earlier report where upregulation of colonic IL-27p28 mRNA was observed in active CrD patients [19] and therefore it is too early to ascribe a clear function to IL-27 in IBD.

Quite unexpectedly in view of the above mentioned immunological observations, ulcerative colitis is associated with almost identical mutations in the *IL23R* and *IL12B* gene regions.[13,20-25]

Psoriasis and psoriatic arthritis

Psoriasis is a common, immune-mediated disorder of the skin, nails and joints with a well-established genetic basis. IL-12p40 and IL-23p19 mRNA are upregulated in lesional psoriatic skin and in addition, antibodies that block IL-12 and IL-23 are effective in psoriasis patients.[26,27] The first genetic data supporting these immunopathological findings in psoriasis were the identification[28] and confirmation[29-32] of a SNP in the 3'UTR of *IL12B*, and later associations with a SNP located 24kb from the 3'UTR, and two SNPs upstream of the 5'UTR of the *IL12B* gene (Figure 3B).[29-31,33,34] In addition, independent missense mutations in the seventh and ninth exon of the *IL23R* gene have been associated with psoriasis (Figure 3A).[29-31,33,34]

Psoriatic arthritis (PsA) is an inflammatory arthritis which develops in roughly one third of the psoriasis patients.The finding that PsA is associated with the same *IL23R* and *IL12B* gene variants confirms the close genetic relation with psoriasis.[35-37] Finally, in both psoriasis and PsA an association was reported with a SNP located 3.7Kb from the 3'UTR of the *IL23A* gene,[34,38] however since this SNP is in fact located in a *STAT2* gene intron a functional association with *IL23A* is questionable.

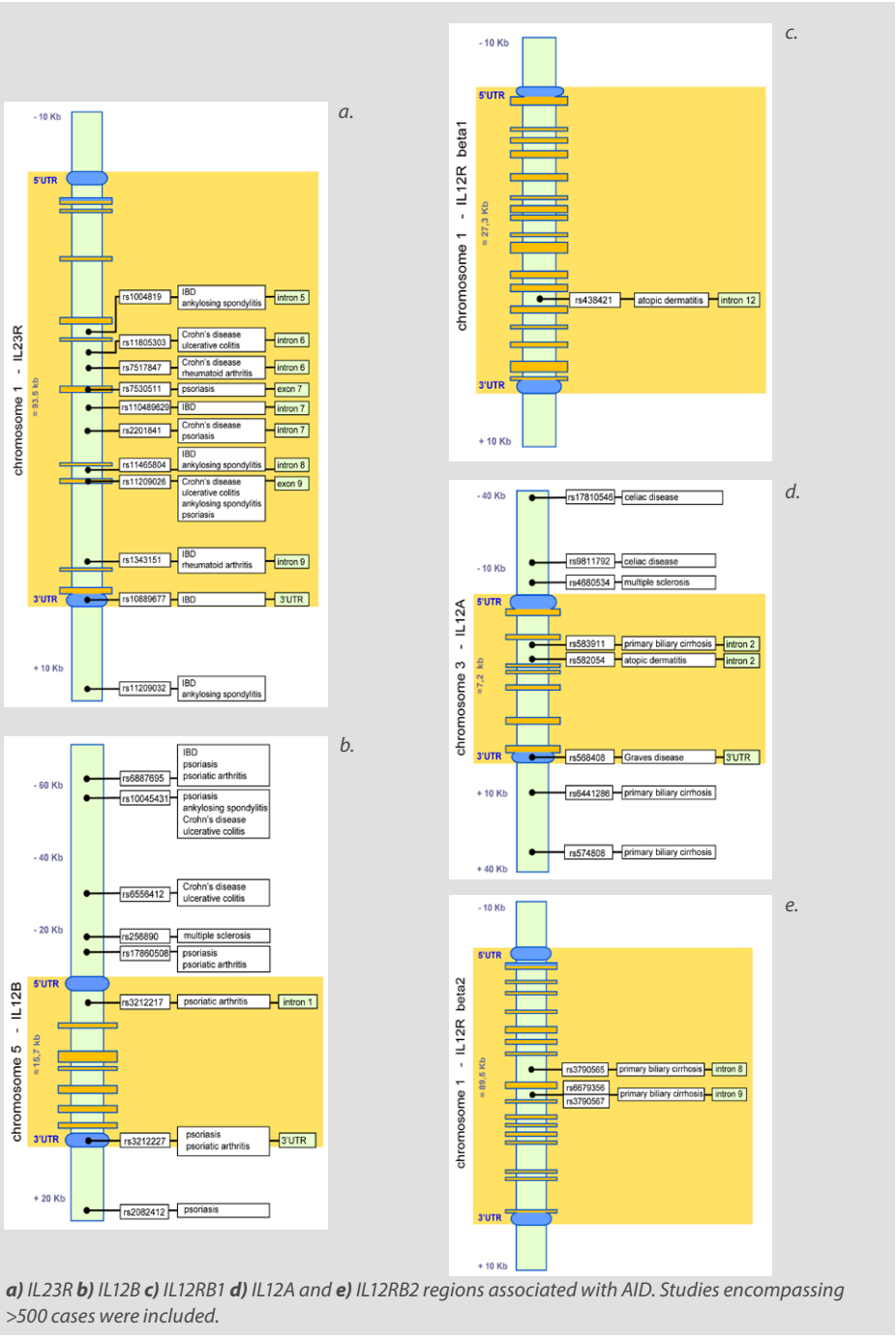
Ankylosing spondylitis

Ankylosing spondylitis (AS) is an inflammatory disease of the sacroiliac joints that is strongly associated with HLA-B27. A role for Th17 cells in the pathogenesis of AS is suggested by increased levels of circulating Th17 cells[39] as well as increased levels of serum IL-17.[40] Apart from a polymorphism approximately 57kb downstream of the *IL12B* gene 3'UTR,[41] AS has been associated with multiple positions within the *IL23R* gene, including several intronic SNPs as well as a polymorphism in exon 9 (Figure 3A-B).[42] The latter association was confirmed in various western populations but remarkably not in three Asian populations,[32,41-49] indicating a population specific effect.

Rheumatoid

Rheumatoid arthritis (RA) is a chronic, systemic inflammatory disorder that primarily affects the synovial joints. HLA-genes account for 30-50% of the overall genetic risk, with HLA-DR1 representing its major association.[50] The observation that IL-17 and IL-23p19 mRNA and protein are present in the synovium of RA patients, point towards a role for Th17 cells as drivers of the immune response in RA.[51,52]

Recent meta-analyses have revealed significant associations with two independent SNPs located in *IL23R* introns (Figure 3A) that were not uniformly identified in the original GWAS.[53,54] Associations between RA and other members of the IL-12 family have not been observed so far.[54-56]



***a)** IL23R **b)** IL12B **c)** IL12RB1 **d)** IL12A and **e)** IL12RB2 regions associated with AID. Studies encompassing >500 cases were included.*

Figure 3 Localisation of SNPs associated with AID in the gene regions encoding the members of the IL-12 family.

THE SECOND CLUSTER: ASSOCIATION WITH TH1 / IL-35

Primary biliary cirrhosis

Primary biliary cirrhosis (PBC) is characterized the destruction of small to medium intra-hepatic bile ducts, leading to chronic cholestasis and eventually cirrhosis. PBC is associated with the HLA-DRB1*08 allele.[57] Immunologically, PBC is characterized by a multi-lineage T-cell and antibody response against the E2 subunit of the pyruvate dehydrogenase complex. While there is evidence for a pathogenic role for IL-12p40 in a PBC mouse model, where deletion of the IL-12p40 subunit resulted in the amelioration of cholangitis[58] these observations have not yet found their counterpart in human PBC.

Despite the lack of immunological data supporting a role for the IL-12 related cytokine family PBC displays strong associations with two SNPs located approximately 15 kb and 19 kb downstream of the *IL12A* 3'UTR, that have been replicated in large studies encompassing multiple populations (Table 1; Figure 3D).[59-61] In addition, in the same studies strong associations with SNPs in the introns of the *IL12Rβ2* gene region were identified (Figure 3E).[59-61] The genetic associations of *IL12A* and its receptor *IL12Rβ2* imply that this cytokine subunit is involved in the pathomechanism of PBC, possibly through IL-12 or IL-35.

Coeliac disease

Coeliac disease (CD) is a gluten-sensitive enteropathy that develops in genetically susceptible individuals. Major histocompatibility class II (MHCII) molecules HLA-DQ2 and HLA-DQ8 are strong genetic risk factors in CD, and account for 35% of the overall genetic risk.[62] The intestinal mucosa of CD patients is characterized by a Th1 profile, with high expression of INF-γ.[63] The role of IL-12 in driving this Th1 response remains elusive. Whereas increased levels of IL-12p35 mRNA have been found in inflamed mucosa of CD patients, IL-12p40 mRNA appears absent.[63-65] Several large GWAS studies, the most recent encompassing over 12000 cases, have revealed strong associations with two SNPs located 10 kb and 40 kb proximal to the startcodon of the *IL12A* gene (Table 1; Figure 3D).[66-69] These associations represent the second strongest non-HLA disease association reported so far.[69] In contrast, no genetic associations with the *IL12B* gene have been found,[69,70] while one study reported a genetic association with the *IL23R* gene but that has not been confirmed yet in large cohort studies.[71]

The association with increased IL-12p35 mRNA expression suggests a functional relationship although formal studies to demonstrate this association are lacking. Since IL-12p40 is absent in the duodenal mucosa of CD patients, it is tempting to speculate that these findings point towards a dysregulated IL-35 rather than a dysregulated IL-12 response in CD.

Multiple sclerosis

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system, resulting in demyelination of the neurons, and consequently to impaired nerve conduction. Autoreactive myelin specific T-cells are more activated than those present in controls and also secrete increased amounts of Th1 cytokines.[72,73] Furthermore, both experimental models

as well as studies in patient specimens suggest that IL-17 producing Th17 cells, and possibly regulatory T-cells, are central in the pathogenesis of MS.[74] The strongest genetic association has been revealed with the HLA-DR*1501.[75] A GWAS including over 8000 cases identified an association with a polymorphism 7,7kb proximal of the *IL12A* 5'UTR gene region that influences MS susceptibility (Table 1; Figure 3D).[76] In addition, a recent follow-up GWAS analyzing almost 10000 cases suggested a SNP approximately 18kb upstream of the *IL12B* 5'UTR to be associated with MS susceptibility (Figure 3B).[77] While these findings may point towards a pathogenic role of this cytokine, functional studies that link these polymorphisms to differences in expression are lacking and in addition the anti-IL-12p40 antibody Ustekinumab failed to induce a clinical response in MS patients.[78]

Graves disease

Graves disease (GD) is an auto-immune thyroid disease with a strong association with the HLA-DRB1*0301 locus.[79] Antibody production against the thyroid stimulating hormone receptor results in thyroid dysfunction in these patients. Based on the production of autoantibodies as well as increased serum levels of IL-4 and IL-5, GD is traditionally considered a Th2 mediated disease. However this picture is far from complete since elevated serum IL-12p40 levels have been found in GD-patients that correlated with free T3 and significantly declined after surgical intervention.[80] In view of these findings it is of interest to note that whereas two large cohort GWA-studies failed to reveal an association with the IL-12 family,[42,81] a smaller study including over 1000 Chinese patients did reveal an association with a SNP in the 3'UTR of the *IL12A* gene (Table 1; Figure 3D).[82]

OTHER AUTOIMMUNE DISEASES

In addition to the two clusters described above that categorize diseases to their respective associations with the IL-12 gene cluster, there are additional immune-mediated diseases that do not show associations strong enough to encompass them into either of the two clusters. Given their high prevalence they are briefly discussed here.

Asthma

Asthma is caused by a Th2 mediated inflammation of the airways. Th2 cells are involved in allergic disorders and protect against extracellular pathogens. There are indications that in this disease a decreased IL-12 secretion results in impaired inhibition of Th2 cell differentiation, leading to an exaggerated Th2 response.[83,84] Yet, IL-12 can also contribute to allergic airway disease as seen in an experimental asthma model.[85] From the genetic perspective multiple small studies have found associations with asthma and the *IL12B* gene region (Table 1; Figure 3B).[86-90] Moreover, a recent study evaluating 197 Chinese asthma patients suggested that a polymorphism in the 3'UTR of the *IL12A* gene is also associated with asthma (Table 1).[91] Nevertheless, none of these associations were found in cohorts involving over 10.000 patients so that it is likely that these observations reflect type I errors.[92,93]

Atopic dermatitis

Atopic dermatitis (AD) is a chronic inflammatory skin disease, characterized by reoccurring typical skin lesions. Similar to asthma, AD is considered a Th2 mediated disease.[94] So far, AD has been associated with an *IL12B* polymorphism,[28] and more recently with variations in the *IL12A*, *IL12Rβ1* and *IL12Rβ2* gene regions (Table 1; Figure 3B-E).[95-97] It should be noted however that these associations were not replicated in a large cohort involving over 5000 patients.[98]

Type 1 diabetes

Type 1 diabetes (T1D) is caused by an autoimmune response towards the pancreatic β-islets, resulting in an impaired insulin production and is strongly associated with HLA-class II molecules.[99] Experimental models have indicated that IL-12 is involved in the pathogenesis of T1D.[100,101] In humans, a polymorphism in the 3'UTR of *IL12B* was associated with T1D susceptibility, and this was linked to a higher IL-12 production.[102] However, results from association studies in other populations have yielded inconsistent results: some confirmed this association,[103-106] whereas others did not.[107-111] A recent report by Morahan and collegues involving almost 3500 patients indicated that *IL12B* polymorphisms might not contribute to overall disease susceptibility.[112]

LIMITATIONS OF GENETIC ASSOCIATIONS STUDIES

During the last decade, significant progress has been made in the identification of genetic risk factors underlying complex immune mediated disorders. In the case of IBD for example, well over 100 distinct genetic risk factors have been identified so far.[11,113] It is therefore not surprising that the individual loci have a limited effect on the trait with relative risks in the order of magnitude of 1.1 to 1.5. This not only hampers identification of such loci (requiring very large data sets), but also challenges the identification of functional consequences of such an association.

The genetic associations identified by GWAS involve associations with particular gene regions, but GWAS are unable to directly identify the culprit gene variant. In fact, it is more likely that another polymorphism in high LD with the tagging SNP is the actual disease causing variant. Fine-mapping of disease-associated regions is a potential method to identify, or at least more accurately localize the culprit polymorphism.[114] This approach has been followed by Tryncka and colleagues, who fine-mapped the *IL12A* gene region in 80 CD patients.[69] These efforts revealed a tight block of highly correlated SNPs, rather than a gradual decay of correlation that would pinpoint the culprit gene variant.[69] This example again underscores the challenges and current limitations of gene identification in complex diseases by the use of GWAS. A technically feasible approach to gain more evidence for a particular genetic association is by linking these data to functional data. This approach involves genome-wide measurement of gene expression profiles using peripheral blood, and subsequent association of these "expression quantitative trait loci" (eQTLs) to SNP data derived from GWAS.[115] As was recently shown in CD,[66] over half of the variants associated with CD are correlated with expression changes in nearby genes suggesting that this approach may be feasible to further zoom in on disease associated SNPs.

A final point that needs consideration here relates to the question whether the polymorphisms discussed affect overall disease susceptibility or, alternatively, whether they are related to specific clinical phenotypes. A hallmark of most autoimmune diseases is the marked clinical heterogeneity and this clinical heterogeneity may reflect underlying genetic and/or pathophysiological heterogeneity. In the majority of GWAS patients are lumped together based on overall diagnosis and a major challenge for future genetic research will be to further dissect these diseases based on their respective clinical phenotypes.

ARE THE IL-12 SNPS SPECIFIC OR SHARED AMONG THE VARIOUS AID?

From the studies reviewed above it has become apparent that a large number of IL-12 related associations exist with a multitude of diseases (Table 1). An intriguing and as yet unanswered question is whether certain polymorphisms are shared among AID so that the presence of a particular SNP simultaneously influences the risk for several AID, or alternatively, that the genes involved are simply key players in the immune response and that any disturbance of the function of this gene, regardless of the precise genetic variant, perturbs the immune response ultimately contributing to disease development. This is of particular interest since the diseases that fit in a particular cluster based on their respective IL-12 related gene associations show considerable clinical overlap. Thus, for instance, it is well recognized that IBD patients suffer more often from sacroiliitis, spondylitis and psoriasis.[116] As an example of such genetic overlap, it can be appreciated from Figure 3A that an association with a missense polymorphism in the *IL23R* (rs11209026) is in fact shared among CrD, UC, AS and psoriasis. In addition, there are several other SNPs in this gene that are associated with more than one AID. As another example, CrD, UC, psoriasis and AS have all been linked to a SNP located upstream of the 5'UTR in the *IL12B* gene region (Figure 3B).

In contrast to the first cluster discussed above where several overlapping associations are found, no shared polymorphisms between AID have been observed so far in the *IL12A* associated Th1 / IL-35 cluster. The only potential overlap is represented by a SNP in the *IL12A* gene region that is associated with CD and another nearby located SNP associated with MS that are in modest linkage disequilibrium (LD: r^2=0.48 in HapMap CEU). The *IL12A* SNPs associated with PBC are not at all in LD with those associated with CD and MS, and therefore most likely represent independent signals.[76]

When reviewing the data from the various studies it is remarkable that SNPs associated with more than one disease can exert different effects on the respective traits. Thus, for example, SNPs in the *IL-23R* gene region (rs7517847, rs1343151) increase susceptibility for RA, whereas they have a protective effect in IBD. Similarly, the previously discussed polymorphisms in the *IL12A* gene region which are in moderate LD and therefore can be considered to be shared among CD and MS also exert opposite effects. It is possible that the polymorphisms exert their effect in distinctive cell types in the respective AID. Alternatively, it can also be hypothesized that the involved cell types (e.g. Th17 cells) exert distinctive roles in the various AID. Functional studies are urgently needed to further answer these fundamental questions.

FROM GENE TO FUNCTION

Majority of disease associated polymorphisms are located in non-coding regions

How do these genetic variations affect normal immune responses to induce autoimmunity? SN polymorphisms can lead to a variety of effects on the transcription and translation of the gene. A polymorphism located in a coding region that alters the protein's amino acid sequence may change the biological function of the protein involved. However, the large majority of disease associated SNPs, including the IL12 associated polymorphisms, are located in introns (≈45%) or in between genes (≈43%) (Figure 3).[117-118] Non-coding SNPs may lead to a decreased or enhanced production of the protein involved by influencing gene transcription in various ways, e.g. affecting enhancers, microRNAs, or long-range transcription regulation.[114] Indeed as discussed in the previous section, a large proportion of genetic risk factors appears to influence gene expression. In addition, evidence is emerging that also synonymous genetic variants may be able to influence protein function. Small translational inaccuracies resulting from such variants may introduce conformational changes in the protein.[119] Intense research addressing these factors is currently ongoing and will hopefully aid in the elucidation of the functional effect of polymorphisms located in between genes. However, as previously discussed, GWAS are unable to exactly identify the disease causing variant so that fine mapping is crucial to localize risk variants that can be used for functional studies.[114] In theory this would also lead to an increased disease association, since the culprit gene is thought to have the strongest association with the disease. Even if fine mapping will not pinpoint the disease causing variant, it will narrow down the number of candidates that should be evaluated in functional studies**.**

Functional consequences of the polymorphisms in the Th17 / Th1 associated disease cluster

As alluded to in the previous sections the *IL23R* gene appears to be the gene with the most consistent associations in the first cluster of autoimmune diseases. The majority of the *IL23R* SNPs associated with these diseases protect against the development of these diseases, suggesting that the presence of these SNPs decreases the function of the proinflammatory Th17 cells. Evidence for this theory has been provided by elegant translational work from DiMeglio and colleagues, who showed that the protective effect of the *IL23R* polymorphism *rs11209026* in psoriasis, PsA, AS and IBD leads to an impaired Th17 effector function.[120] Decreased IL-23 induced STAT3 phosphorylation suggests this is likely duo to hampered IL-23R signaling.[121] The result of this genetic variation is a decreased IL-17 production by IL23R expressing cells.[120-122] Similarly, serum levels of IL-17A in RA patients were lower in those with the minor allele of this SNP.[123]

Another *IL23R* variant (*rs10889677)* located in the 3'UTR is associated with an increased disease susceptibility to IBD. We could recently demonstrate that the risk allele leads to enhanced levels of both IL-23R mRNA and protein. This could be directly linked to a loss of binding capacity for the regulatory microRNAs Let7e and Let7f thereby providing a biological basis for the differences observed.[124]

IL12B is highly conserved displaying little polymorphism in humans.[125,126] Nevertheless, *IL12B* does display genetic variation, but studies to unravel the precise role of these variations have not led to unequivocal results. Several groups have studied the polymorphisms in the 3'UTR region of this gene including the TaqI RFLP polymorphism at position 1188 (rs3212227) which is associated with multiple AID. The A allele was associated with increased IL-12p40 mRNA[102] and increased secretion of IL-12p40,[127] as well as non-different secretion of IL-12p40.[128,129] Subsequent secretion of the biologically relevant IL-12 protein was found to be increased in one study,[128] but decreased in another.[129] Supporting the former is the finding that genetic variations in the coding region of murine IL12B resulted in more efficient binding of the IL-12p40 variant to the IL-12p35 and IL-23p19 subunit, likely due to conformational changes resulting from differential glycolysation induced by the polymorphism.[130]

Remarkably, no clear associations with the *IL23A* gene and any AID have been identified so far. This is likely the result of the exceptional level of evolutionary conservation of the *IL23A* gene, even when compared to the closely related and well conserved *IL12B* gene.[131]

Finally, when interpreting these functional data, it must be realized that some AID show independent associations with multiple SNPs within one gene that may exert opposite effects. A major challenge will be to identify the net result of the various polymorphisms on the expression and/or function of that gene in the various cell types.

Functional consequences of the polymorphisms in the Th1 / IL-35 associated disease cluster

In contrast to the substantial amount of studies that have been performed to address the functional consequences of the *IL12B* en *IL23R* polymorphisms, functional data on the *IL12A* gene region are scarce. Increased IL-12p35 mRNA levels in small intestinal mucosa of CD patients correlate with the rs9811792 risk allele located approximately 10 kb proximal of the start codon of the *IL12A* gene.[132] Another polymorphism (rs582054) located in an *IL12A* intron, is associated with atopic dermatitis and was shown to be inversely correlated with the blood eosinophil count. This finding, together with the decreased IL-12 serum levels in these patients, supports the hypothesis of a decreased IL-12 production leading to a skewed Th1/Th2 profile in dermatitis patients.[96]

At present it is unknown whether an altered *IL12A* gene expression affects primarily IL-12 formation or alternatively with expression of the recently identified IL-35 expression. Answering this question will be crucial to further delineate the precise contributions of the *IL12A* polymorphisms in disease pathogenesis of these AID.

CONCLUDING REMARKS

It is clear that all major autoimmune diseases, perhaps with the apparent exception of type 1 diabetes show genetic associations with the IL-12 gene family. This directly reflects the importance of the IL-12 related genes in T-cell immunity in general, but in addition the differential association that is found for the various autoimmune diseases may shed light on the way these cytokine pathways are involved in the onset and chronicity of the disease. As shown here, the analysis of a single cluster of genes in different autoimmune diseases revealed that these diseases can be distinguished in two major clusters based on their respective genetic associations with the IL-12 gene family. Looking at genetic associations in a broader perspective including multiple diseases may therefore be very helpful to unravel the etiology of an individual disease. The major challenge to further unravel the role of these disease associated gene loci in disease pathogenesis will be to identify the functional consequences of these variants.

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Chapter 11

Coeliac disease associated SNP rs17810546 is located in a gene silencing region

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ABSTRACT

GWAS studies have identified variant rs17810546 in a non-coding region on chromosome 3 as a risk factor for several auto-immune diseases, including coeliac disease. In silico analysis reveals that this variant is located in a transcription regulatory site. By means of reporter constructs we show that this region can override the expression rate of a gene as determined by its native promoter and that this modulation is influenced by the genetic composition of the haplotype which rs17810546 forms with a nearby other variant, rs761008. Secondly, we present data that this genetically imprinted modulation could be involved in coeliac disease through the IL12A gene which is located 40 Kb downstream of this regulatory region. Based on our findings it is most likely that the IL12A gene does so as part of the cytokine IL-35.

INTRODUCTION

Coeliac Disease (CD) is a complex immune mediated disease which is triggered by gluten - a group of storage proteins in cereals such as wheat, barley and rye - in genetically susceptible individuals. CD manifests itself in the small intestine where the duodenal and jejunal mucosa of the patients is infiltrated by lymphocytes and the villi are destroyed, which subsequently leads to malabsorption.[203,204] Eliminating gluten from the diet, leads to restoration of the villi.

A genetic predisposition for susceptibility to CD has been confirmed in many studies. There exists a strong association with the human leukocyte antigen (HLA) class II molecules HLA-DQ2 and HLA-DQ8,[203,205-208] and, although these HLA-molecules are a prerequisite to develop CD, they are not sufficient on their own, as nearly 40% of the general population is carrier of these molecules and only 3% develop CD.[209] It is estimated that the presence of HLA-DQ2 and/or HLA-DQ8 in CD patients accounts for approximately 35% of the overall genetic risk,[210] leaving 65% to other genetic factors to account for.

With the introduction of genome-wide association studies (GWAS) an additional 42 loci for CD outside the HLA region have been identified in the past decade,[211-214] which accounts for another 14% of the genetic variance.[215,216] Next to the identification of these loci, another major finding was that the majority (~93%) of the disease associated SNPs are not localized in the exons of protein coding genes but in non-coding regions.[217-218] Even more so, over 75% of these SNPs are located either in or are in perfect Linkage Disequilibrium (LD) with SNPs in DNase I hypersensitive sites (DHSs).[217] These findings imply that variation in the regulatory elements - which mostly resides outside coding regions - and hence the variation in gene expression, determines for the greater part the genetics of disease etiology.

One of the SNPs with the strongest association in CD, rs17810546, is located on chromosome 3 at 3q25.33, an intergenic region between the genes for SCHIP1 and IL12A. Besides to CD this SNP is reported to be also associated with other auto-immune diseases (AID) i.e. multiple sclerosis, primary biliary cirrhosis, Sjögren's syndrome, systemic lupus erythematosus, systemic scleroderma, and Behçet disease.[218,219] Furthermore a Meta-Analysis by Guo et al. found a highly increased risk for the carriers of the G allele. An increased risk of 82% was reported for GG homozygotes and 37% for AG heterozygotes compared with the common AA genotype.[220] These observations; strong association, multiple associated diseases and high increased risk, prompted us to investigate the region in which rs17810546 resides, in more detail.

MATERIAL AND METHODS

Tissue Samples and RNA and DNA extraction

For the expression studies duodenal biopsies were taken from 29 patients with active coeliac disease (ACD), from 11 patients on a gluten free diet (GFD) and from 16 individuals who underwent endoscopy for polyp surveillance (HC). Specimens were stored at -80 °C until RNA isolation. For total RNA extraction the biopsies were homogenized and RNA was extracted with Trizol (Thermo Fisher Scientific) according to the manufacturer's protocol. Total RNA was quantified by spectrophotometry and the A260/A280 ratio was used to check for possible contaminations. For genotyping, DNA was extracted from stored frozen whole blood samples from 25 of our ACD patients and 6 of our GFD patients. DNA was extracted with the QIAMP DNA blood mini kit, according to the manufacturers protocol (QIAGEN). DNA was stored at – 20 °C until analysis. All patients and controls gave their informed consent and the study protocol was approved by the Medical Ethics Committees from the VU medical center, Amsterdam, The Netherlands.

RT PCR for quantification of gene expression.

cDNA was generated from 2ug of total RNA with the First Strand cDNA Synthesis Kit of Fermentas (Thermo Fisher Scientific) according to the manufacturer's protocol. PCR was performed with 1µL cDNA using the SYBR green method in a Viia 7 real-time PCR system (Applied Biosystems). As the endogenous reference gene we used glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Relative expression was determined by the ΔΔCt method (primers are given in table 1).

Genotyping of the SNP rs17810546 polymorphism

Genotyping for the polymorphism was done by means of RFLP analysis. In short, 50 ng of genomic DNA was PCR amplified using primers that cover the CviQI restriction site when the G allele is present (for primers used see table 1). After PCR amplification, the product was cut with the CviQI restriction enzyme (New England Biolabs) for 3 h at 25 C. After restriction enzyme digestion, products were visualized on an ethidium bromide stained 2% agarose gel showing a 54 bp and 148 bp fragment when G was present, or the uncut PCR product of 203 bp when A was present.

Preparation of allelic rs17810546 constructs and reporter plasmids.

When our hypothesis is right we expect the site of interaction with a regulatory factor at the location of SNP rs17810546. With this in mind we cloned a region of 758 bp surrounding this SNP (position 159,947,063 to 159,947,821 of chromosome 3) from a CC and an AA homozygous ACD patient in the pGEM-T Easy Vector (Promega) between the NheI and XhoI sites, according to the manufacturer's protocol. Sequencing revealed that the clones differed at one other polymorphic site at the location of rs7610082. Three of the four possible haplotypes were obtained from patient material, the lacking G_A haplotype was generated from the A_A

Table 1 Primers used in the study.

Primer	Sequence
Forward typing primer	TGCTAGGCAGATCAAGAACCCAACA
Reverse typing primer	AGTGTAGCCATCTGAGCAGGCA
Forward NheI cloning primer	CTCGCTAGCTGGATACCCTGAGACAACACA
Reverse XhoI cloning primer	CTCCTCGAGGACTAAGTCATCAAGCACAGGA
Forward internal G mutation primer	CCTGTTTAGACATC**G**TACCACAGAAAGC
Reverse internal G mutation primer	GCTTTCTGTGGTA**C**GATGTCTAAACAGG
Forward KpnI cloning primer	CTCGGTACCTGGATACCCTGAGACAACACA
Reverse HindIII cloning primer	CTCAAGCTTGACTAAGTCATCAAGCACAGGA
Forward IL12A primer	TCCTCCTTGTGGCTACCCTG
Reverse IL12A primer	TTTGGGAGTGGTGAAGGCAT
Forward IL12B primer	CGGTCATCTGCCGCAAAAAT
Reverse IL12B primer	GATGCCCATTCGCTCCAAGA
Forward EBI3 primer	GCAGCAGACGCCAACGT
Reverse EBI3 primer	CCATGGAGAACAGCTGGACAT
Forward p28 primer	CCTGGTTCAAGCTGGTGTCT
Reverse p28 primer	TGGAAGGTCAGGGAAACATC
Forward GAPDH primer	CCATGTTCGTCATGGGTGTG
Reverse GAPDH primer	GGTGCTAAGCAGTTGGTGGTG
Forward IFN g primer	AGAAACGAGATGACTTCGAAAAGC
Reverse IFN g primer	GGCGACAGTTCAGCCATCA
Forward BCL6 primer	TGGACTGTGAAGCAAGGCAT
Reverse BCL6 primer	ATGGCGGGTGAACTGGATAC
Forward RUNX1 primer	CACCCTGGAGATGTTAAGGCAG
Reverse RUNX1 primer	GTGCATTCTGAGGGCTGTCAT
Forward RUNX3 primer	TGGCCGTCTCATCCCATACT
Reverse RUNX3 primer	CACTGGGCATAGCTGGAGAC
Forward IL12A-AS1	CGAGCGAGCCAAAGACCTG
Reverse IL12A-AS1	GGCAAGATAAGCCCAACTGC

With the mutation primers, the A to G converting nucleotide is shown in bold.

haplotype by means of recombinant PCR, using the forward and reverse cloning primers together with the specific internal mutation primers (table 1)[221] in which the A nucleotide is specifically replaced by the G nucleotide ensuring that the constructs used, would only differ at the SNP sites. The four sequences were subsequently cloned between the KpnI and HindIII site of the reporter construct pGL4.23[luc2/minP] Vector (Promega), upstream of the minimal promoter in front of the luc2 gene (for sequences see table 1)

siRNA-induced Gene Silencing and Luciferase Assay

Hek293T-cells were plated in 24 wells plates (at $0.5x10^5$/well) in DMEM (Thermo Fisher Scientific) p/s 10% FCS. The next day, at 80% confluency, cells were transfected for 48 h. in DMEM containing the appropriate transfection mixtures without antibiotics.For determining the optimal siRNA

concentrations; transfection mixtures/well consisted of 500 µL DMEM 10% FCS w/o antibiotics, 1µL Dharmafect 1 as transfection reagent (Thermo Fisher Scientific) and 50, 75 or 100 nM of specific targeting siRNA (ON-TARGETplus Human BCL6 siRNA L-011591-01-0005, SMARTpool: ON-TARGETplus RUNX1 siRNA L-003926-00-0005, SMARTpool: ON-TARGETplus RUNX3 siRNA L-012666-00-0005 (GE Healthcare)) or the same concentrations of a non-targeting negative control RNA (ON-TARGETplus Non-targeting Pool D-001810-10-05 (Thermo Fisher Scientific)).

Transfections with reporter constructs included 500 ng of reporter plasmid together with 10 ng of a Renilla Luciferase [222] plasmid per transfection next to the specific targeting siRNA or non-targeting negative control RNA. DharmaFECT Duo Transfection Reagent T-2010-01 (Thermo Fisher Scientific) was used as transfectant. Transfections were performed according to the manufacturer's protocols and included transfections with empty vector that served as the negative reference control.

Luciferase activities were measured at 48 h. post transfection, using the dual luciferase system (Promega) according to the manufacturer's protocol. The ratios of firefly luciferase signal to Renilla luciferase signal were used to normalize firefly luciferase activity and account for differences in transformation efficiency.

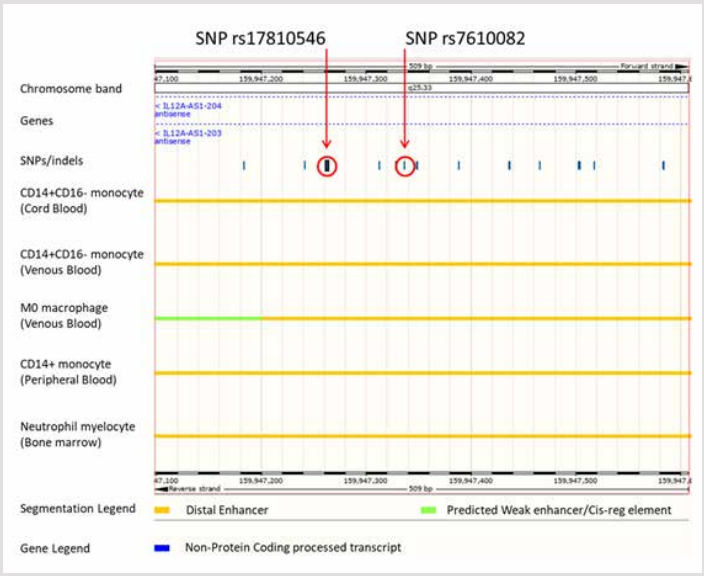
Statistical analysis and datamining

Unless otherwise stated the Mann-Whitney U test was used for statistical evaluation using Prism version 7.00 for Windows, GraphPad Software, Inc. (http://www.graphpad.com).
Gene level analysis was done with Ensemble Genome Browser (Genome Assembly GRCh38.p12) http://www.ensembl.org/Homo_sapiens/Info/Index. Phylogenetic foot printing was performed with the online tool RelA (http://www.bsc.es/cg/rela/home.php) and haplotype analysis with the LDhap module from the LD link website (https://analysistools.nci.nih.gov/LDlink/).

RESULTS

SNP rs17810546 is located in a transcription regulatory region.

Inspection with the Ensemble Genome Browser (Genome assembly: GRCh38.p12 release 95) (http://www.ensembl.org/Homo_sapiens/Info/Index) revealed that SNP rs17810546 is located in distal enhancer sites of a number of immune regulatory cells known to transcribe the IL12A locus: monocytes, macrophages and neutrophils (figure 1A).



(a) *SNP rs17810546 is located in a in transcription regulatory region (features extracted from Ensemble assembly: GRCh38.p12 with the segmentation feature within the gene regulation section turned on. SNPs are shown as vertical blue lines; SNP rs17810546 and SNP rs7610082 are marked with red open circles. The intronic region of IL12A-AS1 is given as a blue dotted line*

(b) *Quantitative expression of IL12A-AS1 in duodenal biopsies from CeD patients (ACeD and GFD) and healthy controls (HC). RNA was isolated and expression of IL12A-AS1 was analyzed by Q-PCR. Expression was normalized against the expression of the house-keeping gene GAPDH (ns = non-signficant).*

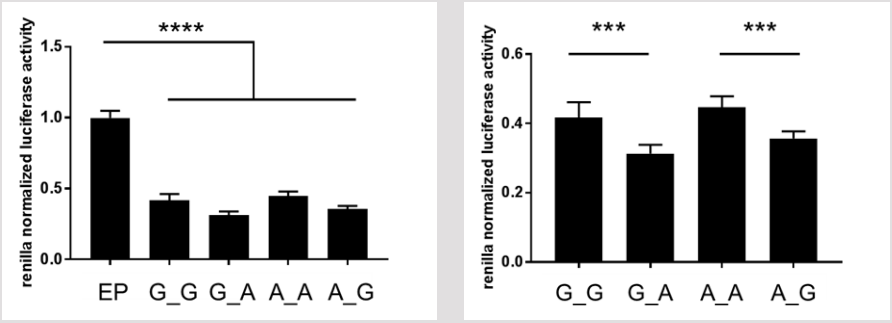
(c) *Organization of the four haplotypes used in the Luciferase reporter experiments.*

Figure 1.

These sites are classified as such with the segmentation algorithms, algorithms that partition the genome into regions with distinct epigenomic profiles. These are genomic regions of similar signal patterns over a selected number of assays. The location in such regions make it conceivable that a SNP can alter the recognition site of a transcription factor. And as the proper binding of transcription factor's is the major determinant of when and where genes will be expressed[223] this could lead to a compromised regulation of gene expression. What figure 1A also shows is that the SNP is located in an intronic region of the long non coding RNA gene IL12A-AS1 .

IL12A-AS1 is not expressed in Duodenal tissue

As long non-coding RNAs are reported to be often involved in transcriptional regulation,[224] we investigated whether the presence of SNP rs17810546 influences the expression of the non-coding antisense transcript of IL12A-AS1 and in this way indirectly the expression of the IL12A gene, we determined its expression in our samples by means RTPCR. As is shown in Figure 1B transcriptional activity is sporadically present above background. A finding that is corroborated in the "EMBL-EBI Expression Atlas" which does not find expression for this gene in duodenal tissue.



a. *b.*

Hek 293T-cells were transfected with either empty plasmid (E P) which served as control for base line activity or with plasmids containing one the four possible haplotypes (G_G, G_A, A_A, A_G) of the cloned region. Columns represent Renilla normalized luciferase activity of the 4 plasmids compared to the luciferase activity of the empty plasmid which was set at 1.Transfections were performed in sextuplicate; each measurement was performed in triplicate; bars represent mean +/- SD.

Figure 2.

(a) *Repression of luciferase activity by the presence of the putative regulatory region upstream of the IL12A gene. (**** P<0.0001 compared with EP by 1-way ANOVA followed by Dunnett's multiple comparison test).*
(b) *Luciferase activity is influenced by the haplotypes formed by genotypes of rs17810546 and rs9811792 in the cloned DHS region. (***p<0.001, 1-way ANOVA followed by Tukey's multiple comparison test).*

SNP rs17810546 in combination with SNP rs7610082 influences expression of gene activity. To explore the nature of this region further we cloned a stretch of 500 bp surrounding SNP rs17810546 (comprising position 159,947,098 to 159,947,606 of chromosome 3) into the pGL4

reporter vector. DNA was extracted from a homozygous individual for the G allele and a subject homozygous for the A allele. Sequencing revealed that besides the anticipated differences at the rs17810546 locus, our clones also showed variation at 86 bp 3' downstream at the locus for rs7610082 (allele frequency A:0.179 G: 0.821) (figure 1C). Three out of the four possible haplotypes (figure 1C) were derived from donor material; the fourth was obtained by means of mutational PCR (see materials and methods). The constructs were transfected in the HEK293T-cell line and the presence of possible regulatory properties of the cloned sequence was assessed by measuring the activity of the reporter luciferase gene (figure 2). What is evident from figure 2A is that the presence of the cloned region has a major down-regulatory effect on the expression of the luciferase gene (about 50% as compared to the base line expression seen with the empty plasmid). This is indicative that the cloned region contains sequences to which transcription down-regulatory factors can bind.

Figure 2B shows the fluorescence emitted by the constructs while in the repressed state. What is evident from the residual luciferase activity of the repressed gene is that while it is influenced by its genetic composition it is rather so by the haplotype formed by both SNPs than by each individual SNP .

Identifying possible binding factors by means of phylogenetic foot printing.

To identify possible transcription factors whose recognition sites overlap the sites of the SNPs and in this way would be influenced in their binding capacities we employed phylogenetic foot printing. This method relies on identification of regions of high sequence conservation between different genomes as these conserved regions are likely to contain important regulatory sites.[225] For our search we employed the online tool ReLA [226] (http://www.bsc.es/cg/rela/home.php). The 11 orthologue sequences with which we compared our sequences (G_G or A_A) were all mammalian and are given in table 2A.

Table 2a.

Orthologue sequence from	Distance in bp from IL12A gene
Camelus bactrianus	37855 bp at 3' side
Ursus maritimus	32877 bp at 5' side
Oryctolagus cuniculus	49950 bp at 3' side
Lipotes vexillifer	41566 bp at 3' side
Equus caballus	50681 bp at 3' side
Orycteropus afer afer	72228 bp at 3' side
Samira boliviensis boliviensis	40228 bp at 5' side
Balaenoptera acutorostrata	41672 bp at 3' side
Canis lupus	36417 bp at 3' side
Pteropus alecto	41168 bp at 5' side
Sus scrofa	47794 bp at 3' side

Shown are the species of which orthologue's of ENSR00000161164 were used in phylogenetic foot-printing. Also is given the distance in bp from the 5' or 3' side of the IL12A gene.

We ran the application loaded with the orthologue sequences against the A_A or G_G as reference sequence. This resulted in the identification of 4 transcription factor's that could have differential binding capacities depending on the allele present at its binding site (table 2B).

Table 2b Results of phylogenetic foot-printing.

	Haplotype G_G			Haplotype A_A		
	conserved	**Transfac id**	**name**	**conserved**	**Transfac id**	**name**
rs17810546	-	-	-	1	M00039	CREB
	-	-	-	1	M00776	SREBP
	-	-	-	1	M00769	RUNX
	Haplotype G_G			**Haplotype A_A**		
	conserved	**Transfac id**	**name**	**conserved**	**Transfac id**	**name**
rs7610082	2	M01183	BCL6	-	-	-

11 orthologue sequences were screened for conservation of transcription factor binding sites. After this primary screen it was investigated which conservation was retained/abolished when the analysis was rerun in the presence of the haplotypes G_G or A_A. transcription factor's identified in this way would be plausible candidates for the differential binding seen in figure 3B. (Transfac id = Transcription Factor Matrix Id as is used by TRANSFAC (TRANScription FACtor database), a database of eukaryotic transcription factors [261]

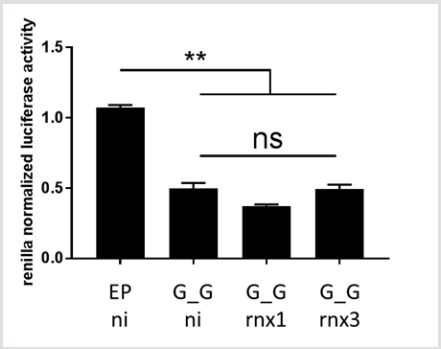
When the G allele of rs17810546 is present, the binding potential for transcription factor's CREB, SREBP and RUNX could be compromised and the same applies to BCL6 when the A allele of rs7610082 is present.

To investigate this further we choose BCL6, RUNX1 and RUNX3 as the most likely candidates to be involved because of their reported down regulatory properties and their role in the generation of regulatory T-cells[227,228] and lymphocyte differentiation[229-235] processes important in immune-mediated diseases .

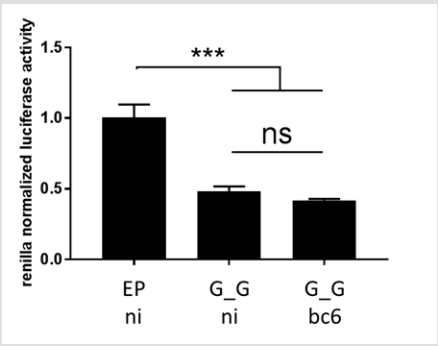
Retained repression of luciferase activity despite abrogated expression of the putative down-regulatory factors by means of specific interfering small RNA's.

We investigated the possible involvement of the transcription factors BCL6, RUNX1, RUNX3 – or combinations of these three – on the repression of the reporter gene in our constructs, by silencing them with siRNA's. For reasons of efficiency we restricted our co-transfections of reporters together with a-specific non-interfering RNA (ni) or specific interfering siRNA's (rnx1: RUNX1, rnx3: RUNX3, bc6: BCL6) to the G_G construct with the intention of expanding our experiments to the other plasmids if justified by these preliminary experiments. Transfection with siRNA's led to a reduction of about 80% of the targeted mRNA's, however this did not resolve the repression of luciferase activity seen with the G_G construct as compared to the luciferase activity seen with the empty plasmid (figure 3). The EP column remains significantly elevated in comparison with all the G_G transfections. To exclude the possibility to miss a significant but modest reversal of luciferase activity, we included transfections of the G_G construct with non-interfering RNA. As can be seen in figure 3 (A, B, and C) no such reversal

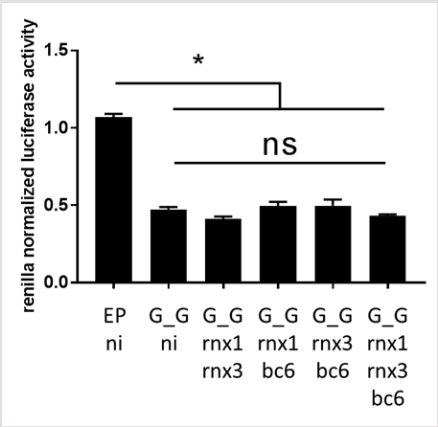
could be seen at a statistical significant level, indicating that the Luciferase repression concerns involvement of other transcription factors or combinations thereof, or other as yet unknown (co)factor(s).



a.



b.



c.

Luciferase activity is not restored to the basic transcription level seen with the EP; compare EP ni with G_G transfection combinations. Also not even a significant slight de-repression at the level of the regulatory region containing plasmids themselves could be discerned, compare G_G ni with varies combinations if specific siRNA's.

***(a)** Luciferase activity after co-transfection with non-interfering RNA or specific RUNX1 or RUNX3 siRNA. **(b)** Luciferase activity after co-transfection with non-interfering RNA or specific BCL6 siRNA. **(c)** Luciferase activity after co-transfection with non-interfering RNA or combinations of specific RUNX1, RUNX3 or BCL6 siRNA.*

Hek 293T cells were transfected with either empty plasmid (EP) or the plasmid containing the G_G haplotype. Transfections were done for 48 hours in the presence of a control non-interfering RNA (ni) or specific siRNA's (rnx1: siRNA for RUNX1, rnx3: siRNA for RUNX3 and bc6: siRNA for BCL6. Concentrations used were 75 nM with the single ni or si transfections – panel A and B or 100 nM with the combined transfections panel C)

*Columns represent Renilla normalized luciferase activity of the G_G plasmids compared to the luciferase activity of the empty plasmid which was set at 1. (*** P<0.001, ** P<0.01, * P<0.05, ns statistical not significant. Kruskall Wallis was used for comparison with EP ni which was followed by Dunnett's multiple comparison test when comparing G_G ni with the other G_G transfections.*

Figure 3.

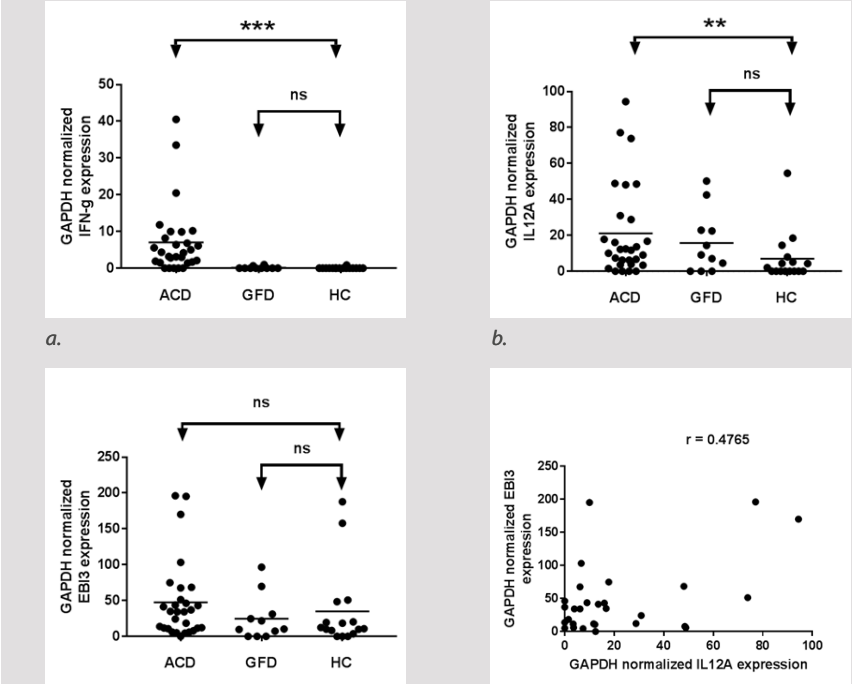
IL12A expression is upregulated in active CD and significantly correlated with EBI3 expression indicating a role for IL-35 in CD

Next we wanted to investigate whether the presence of the variant has an influence on the expression of the IL12A gene in CD. As mentioned before the variant is located between two genes i.e. IL12A and SCHIP1. The expression of SCHIP1 was shown to be influenced in an allele specific way by the presence of SNP rs17810546, with higher expression in the presence of the G allele.[236] However, as regulatory elements can act on multiple genes (for an overview see[237], we opted for the IL12A gene because of its prominent role in the immune system and hence its possible role in auto-immune diseases. The IL12A gene product cannot exist on its own[238] but instead is only known to be secreted combined with one of its co-constituents – IL12B or EBI3 – to form the hetero-dimeric pro-inflammatory cytokine IL-12p70 or the anti-inflammatory cytokine IL-35, respectively.[239-242] This leaves the question in which capacity - as part of IL-12p70 or as part of IL-35 - the IL12A gene can be involved in higher susceptibility for CD, and whether its expression could be linked to the genotype of rs17810546. To investigate this we isolated RNA from duodenal biopsies from 29 patients with active coeliac disease (ACD), from 11 patients on a gluten free diet (GFD), and from 16 healthy controls (HC) and measured the expression of IL12A, IL12B and EBI3 by means of Q-PCR. As EBI3 can also join with p28 to form the IL-27 cytokine[243] we also investigated the expression of the p28 gene. Furthermore, to verify the presence or absence of inflammation, we also measured the expression of IFNγ. Figure 4A shows that IFNγ is abundantly expressed in the ACD group, indicating an ongoing inflammation, which is absent after a gluten free diet in the GFD group. Expression of IL12A is strongly upregulated in ACD patients and to a minor extent in GFD patients as compared to the HC group (figure 4B). Figure 4C shows that in duodenal biopsies EBI3 is abundantly expressed in all three patient groups and that this expression in the ACD group is significantly correlated with the expression of IL12A (figure 4D) which is suggestive for co-regulation between both genes. At the same time however measurable expression of the other possible partners was either absent (IL-27 p28) or very sporadic (3 out of the 55 subjects) for the IL12B subunit. This absence of possible other co-partners together with the observed co-expression of both genes is highly suggestive that IL-35 is involved in CD. We next investigated whether this elevated expression could be linked to the genotype of rs17810546. DNA was available from 25 ACD and 6 GFD patients of our cohort. Genotyping was done by means of RFLP. Results are shown in table 3.

Table 3 Combined results of the genotype for SNP rs17810546 and expression of the IL12A gene in 31 Coeliac disease patients (25 with active coeliac disease and 6 on a gluten free diet)

Risk allele present	N	Median IL12A expression
+	9	16.74
-	22	10.44

Although a trend to a higher expression in the presence of the risk (G) allele is present it did not reach statistical significance. This is most probably due to the low frequency of the risk (G) allele in the European population (allele frequency G: 0.094 A: 0.906 (1000 Genomes project)).



Quantitative expression of IFNγ, IL12A and EBI3 in duodenal biopsies from CeD patients (ACeD and GFD) and healthy controls (HC). RNA was isolated and expression of IFNγ, IL12A and EBI3 was analyzed by Q-PCR. Expression was normalized against the expression of the house-keeping gene GAPDH

(a)** Expression of IFNγ (p<0.001, ns = non-significant). **(b)** Expression of IL12A (** P < 0.01, ns = non-significant). **(c)** Expression of EBI3 (ns = non-significant). **(d)** Correlation diagram in ACeD patients of the IL12A expression (x-axis) vs the EBI3 expression (y-axis). Pearson's correlation coefficient r = 0.4765 (P<0.009, 95% CI 0.13 to 0.71)*

Figure 4.

DISCUSSION

Because of its reported associations with multiple AID's we investigated in this study in more detail the region in which SNP rs17810546 resides. To this end we cloned a region surrounding the SNP in reporter constructs which revealed that rs17810546 is situated in a regulatory element with gene-expression repressive properties and that these properties are modulated dependent on the genotype of rs17810546. Phylogenetic foot printing identified possible candidate transcription factor's of which binding would be compromised by the presence of the SNP, however RNA interfering experiments did not corroborate this. Nonetheless it is clear that the risk locus which harbors rs17810546 has the characteristics of an expression quantitative trait locus (eQTL),[244] an observation also reported by Plaza-Izurieta et al..[236] They showed an allele specific effect for rs17810546 on the expression of SCHIP1, a gene which has its transcription start site about 675 kb upstream of this SNP. This observation, and ours, are in contrast with that of Ricaño-Ponce and Wijmenga who, in 2013, reported for CD that 14 out of 26 identified risk loci showed an eQTL effect, but did not detect such an effect for rs17810546.[245] This discrepancy could very well lie in the tissue used for these experiments. eQTLs appear to be highly cell-type–dependent and it is recommended to use cells or tissues directly involved in the pathophysiological process [246] and where we and Plaza-Izurieta et al. indeed used the target tissue for CD i.e. intestinal biopsies, Ricaño-Ponce and Wijmenga relied on cells obtained from peripheral blood.

Next we investigated whether the increased risk whit the G allele present can be mediated through the IL12A gene in its capacity as a constituent of IL-35, a member of the IL-12 family to which in mouse models of AID's anti-inflammatory properties are ascribed.[239,247-250]

In duodenal biopsies taken from CD patients with active disease we found a significant elevated expression of the IL12A gene compared to the expression levels found in healthy controls. This elevated expression was strongly correlated with that of EBI3, the counterpart with which the IL-12p35 subunit forms active IL-35. This elevated expression of both these constituents together with the absence of the other counterparts i.e. IL-12p40 for IL-12p35 or IL-27p28 for EBI3 is strongly indicative for a role of IL-35 in CD (for an oversight of the IL-12 family of cytokines see[251]).

That a genetically determined higher synthesis of an alleged immunosuppressive cytokine can be involved in susceptibility to an inflammatory disease seems puzzling, however, the biological role of the new members of the IL-12 cytokine family in inflammation can be dual, IL-27 has both pro-inflammatory (Th1), as well as anti-inflammatory functions[252] and also for IL-35 pro-inflammatory properties have been reported. In mice IL-35 exacerbated Lyme arthritis in Borrelia-vaccinated and -infected mice,[253] and in a collagen-induced arthritis model IL-35 therapy led to a Th17/Treg imbalance in favor of the pro-inflammatory Th17 population aggravating the arthritis.[254] Also in humans pro-inflammatory properties are reported: Filková et al. showed increased expression of both IL-35 (p35/EBI3) subunits in rheumatoid arthritis (RA) synovial tissue. Such a scenario could also be at play in CD. A relevant observation in this respect is that IL12A and EBI3 are still abundantly expressed in biopsies of our patients on a GFD

while the inflammation has disappeared as is exemplified by the absence of IFNγ. Furthermore, expression of IL12A and EBI3 has been found in tissue from healthy controls. This indicates that the presence of IL-35 per se is not enough to provoke an inflammatory response in the target tissue but instead seems more indicative for a role in normal gut homeostasis. On the other hand, its stark upregulation in ACD indicates an involvement in an inflammatory process. It looks as if, at least in CD, there is a threshold above which it shows its pro-inflammatory features. This switching in properties could be reached via differential upregulation of the IL-35 receptor chains - IL-12Rβ2 and gp130 - in different cell types, depending on the level of IL-35 present. This possibility is supported by the observation that IL-35 itself can induce expression of pro-inflammatory molecules in mononuclear cells which express both subunits of the IL-35 receptor.[255]

Of relevance in assigning an anti or a pro-inflammatory role to IL-35 seems to lie in the tissue chosen. When comparing serum from patients with active systemic lupus erythematosus, immune thrombocytopenia, rheumatoid arthritis (RA), Sjögren syndrome and inflammatory bowel disease (IBD) with serum from healthy controls, lower levels of IL-35 were reported in patients.[256-260] It is this reciprocal correlation that led to assigning an anti-inflammatory role to IL-35. However when using tissue from the site of inflammation itself this picture is reversed. In RA IL-35 is upregulated in the synovium.[255] Likewise in IBD over-expression was seen in colonic tissue.[257] And also our study shows upregulation of IL-12p35 and EBI3 subunits in duodenal biopsies from patients with active disease. When considering that the site of inflammation most probably reflects more aptly the dynamics of a full blown inflammation, we conclude that in active CD IL-35 displays a pro-inflammatory role in the same way as was earlier demonstrated in RA.[235]

CONCLUSION

In conclusion, we present evidence that SNP rs17810546 is located in an expression regulatory region and influences expression in a genotype dependent fashion.

Furthermore we make it plausible that the upregulated expression of the nearby situated IL12A gene is involved in the pathogenesis of CD in its capacity as a constituent of the cytokine IL-35, which displays pro-inflammatory properties in CD. Finally we propose, in keeping with the reported anti- as well as pro- inflammatory properties of IL-35, that in CD IL-35 has a threshold level above which it switches from an anti-inflammatory to a pro-inflammatory cytokine. Whether such is the case and if so, how and why this is the case, needs further investigation.

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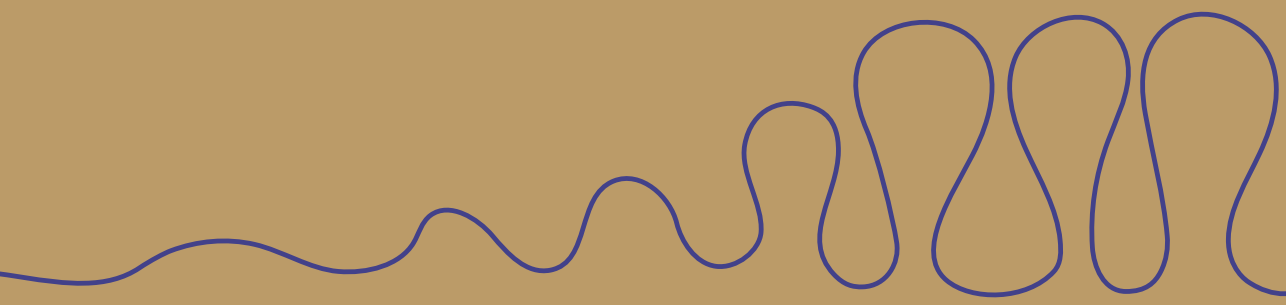
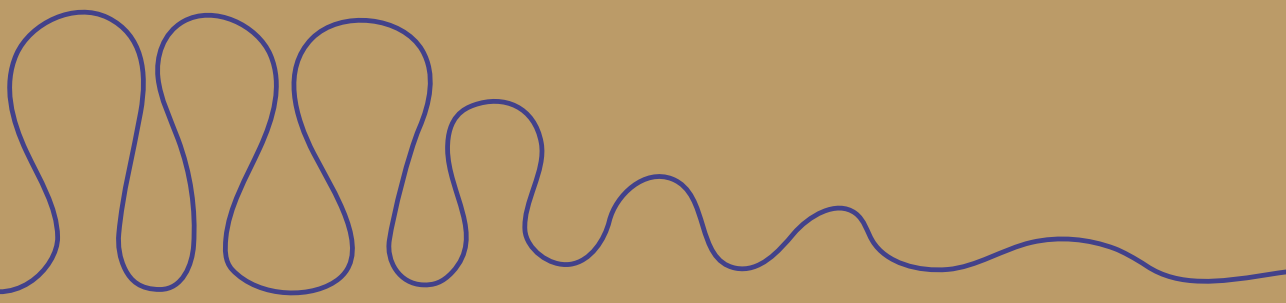
PART IV

SUMMARY, DISCUSSION AND FUTURE PERSPECTIVES





SUMMARY, DISCUSSION AND FUTURE PERSPECTIVES

The current thesis reports on novel insights in the pathogenesis, diagnostic developments and clinical aspects of (refractory) coeliac disease. It further provides an overview of other immune-mediated enteropathies with a focus on adult-onset autoimmune enteropathy. The novel insights will be summarized and discussed per chapter.

SUMMARY AND DISCUSSION

Chapter 1. *Immune-mediated enteropathies: from bench to bedside*

This first chapter provides an overview of immune-mediated enteropathies and discusses clinical, diagnostic and therapeutic aspects. There is considerable overlap between various immune-mediated enteropathies in clinical, histological and serological features. This means further research is needed to reveal the exact link and possible partly shared pathogenesis between them, but it also underlines the challenge it sometimes imposes in clinical practice to differentiate between these enteropathies, which is important to determine optimal therapy and prognosis.

Chapter 2. *Outcome of referrals for non-responsive coeliac disease in a tertiary center: low incidence of refractory coeliac disease in the Netherlands*

Previous studies showed inconsistent results regarding the prevalence of refractory coeliac disease (RCD) in Europe and North-America, which has led to much debate about population characteristics, diagnostic workup and definition of RCD.[1] Our data show that RCD is indeed an extremely rare disease, with a slightly higher prevalence of RCD type 1 over RCD type 2, and an incidence of RCD in the Netherlands that is more similar to other European and North-American populations than previously thought.

In CD patients that fail to improve upon a gluten-free diet (GFD), RCD was responsible for these persisting symptoms in only 23%. In a similar percentage of patients the symptoms were caused by inadvertent gluten ingestion. Others have found similar high percentages of inadvertent gluten ingestion responsible for persisting symptoms, and our data shows it currently is still a common problem which underlines the need to refer patients that fail to respond to the GFD to an expert dietitian in an early stage and carefully review the dietary approach.[2,3] In one third of patients that fail to respond to a GFD duodenal mucosa recovered completely, and in some of these patient diseases associated with CD such as microscopic colitis or, to a lesser extent, inflammatory bowel disease were found. The majority of these patients were however diagnosed with irritable bowel syndrome, which indeed appears to be more common in CD patients, even when following a GFD.[4] Whether these symptoms are related to inadvertent minor gluten intake, undetected by our current serologic tests and dietary review, or whether CD patients are prone to IBS is unclear. In patients with persistent symptoms and duodenal abnormalities despite a GFD, one has to be aware of an alternative diagnosis other than (R)CD, such as autoimmune enteropathy, common variable immune deficiency disorder (CVID) or olmesartan-associated enteropathy (OAE), in which patients will also not respond to dietary

measures. In our study this was only the case in a minority of patients, probably due to strict inclusion criteria in which the original diagnosis of CD was revised based on serological tests, HLA-genotype and histological findings. It is not unlikely that this is an underestimation of patients with an enteropathy other than CD that in daily clinical practice would be erroneously classified as CD.

Chapter 3. *Adult onset autoimmune enteropathy: limited need for long-term immunosuppressive therapy*

Adult-onset autoimmune enteropathy (AIE) is extremely rare and therefore much is unknown about this disease. This chapter describes clinical, serological and histological features of thirteen adult onset AIE patients that have been diagnosed at our center over a 13 year timespan. While this study underlines the rarity of the disease, it is the second largest case-series of adult AIE described so far. Patients in our cohort were more often female (62%), median age at diagnosis was 52 years (range 23-73), and 46% had previously been diagnosed with an autoimmune disease. In 85% circulating auto-antibodies were found, again underlining the susceptibility for autoimmunity in these patients. The coeliac disease associated HLA-DQ 2.5 haplotype was overrepresented in our cohort (79% vs general population 35%) while CD had been excluded, which draws attention to the role of HLA-DQ 2.5 in non-CD enteropathies given it has also been associated with olmesartan-associated enteropathy. The latter mimics the histological abnormalities found in AIE, and anti-enterocyte antibodies (AEA) are found in up to 30% of patients.[5] Histologic features of AIE encompass active chronic enteritis, deficiency of goblet and Paneth cells, as well as apoptotic cells, and histological patterns can vary between patients and therefore have been divided into four subtypes.[18] AEA are associated with AIE, but are no longer absolutely required for the diagnosis AIE according to the modified AIE criteria.[6] The role of anti-enterocyt antibodies (AEA) in the pathogenesis of AIE remains unclear. In agreement with previous studies, the levels of AEA in our study did not correlate with the degree of villous atrophy nor clinical presentation, suggesting AEA's to be secondary to T-cell mediated intestinal tissue damage that leads to release of (auto)antigens that can lead to (auto)immune response by (auto)antibody production.[7] For this purpose we tested AEA's in other patients with enteropathies with various severity. We found no AEA's in healthy controls, but in patients with an enteropathy these antibodies could be identified in a minority and even more so, presence of AEA's appeared to increase with the severity of the enteropathy, again supporting the idea of being a secondary phenomenon. Adult-onset AIE shows to be a severe disease as it requires hospitalization for intravenous fluid and potassium suppletion in more than half of the patients, and total parenteral feeding in more than two thirds of patients. Three patients (23%) died as a result of therapy-refractory malabsorption. Steroids induced clinical remission in 62% of patients. Maintenance therapy with thiopurines or budesonide was effective in these patients. When patients were refractory to steroids several therapies showed only moderately effective. One patient with severe therapy-refractory AIE was eventually successfully treated with autologous stem cell transplantation and has been clinically well ever since, without medication for over 10 years now. Remarkably, three other

patients are in a long-lasting drug-free clinical remission after being treated for 3-7 years with immunosuppressive therapy, which shows that adult-onset AIE is curable in some patients.

Chapter 4. *Novel variant of EATL evolving from γδ T-cells in a RCD patient*

This chapter describes a patient with RCD type 1 that developed a novel variant of an enteropathy-associated T-cell lymphoma (EATL) originating from mucosal γδ T-cells in the duodenum. Typically, patients with RCD type 2 harbor premalignant monoclonal αβ T-cells with an aberrant phenotype that are located in the small intestine, and these patients are at risk to develop an EATL. Numbers of γδ T-cells are elevated in the intestinal mucosa of CD patients compared to healthy controls, during both active disease and after recovery upon a GFD. Their exact role remains unclear, but despite their increased activity in CD malignant transformation has not been reported in the literature so far. In contrast, peripheral γδ T-cells lymphoma's are well known especially in hepatosplenic T-cell lymphomas, but our patient had dissimilar clinical, phenotypical and chromosomal characteristics. This unique case of EATL with distinctive features and originating from mucosal γδ T-cells, together with another recently described new EATL variant that arose in an AIE patient, underlines the need for a new classification for EATL, and raises awareness of EATL development in these patients.[21]

Chapter 5. *Optimal strategies to identify aberrant intraepithelial lymphocytes in refractory coeliac disease*

Clonal intraepithelial lymphocytes with an aberrant phenotype are the hallmark of RCD type 2. These cells are thought to be the precursor cells from which EATL originate, which occurs in about 50% of RCD type 2 patients.[8,9] Accurate identification of this premalignant cell population is therefore crucial in order to timely initiate aggressive therapy such as cladribine, and for those eligible, autologous stem cell transplantation.[10,11] Phenotypical analysis using flow cytometry has previously been shown to be superior to TCRG clonality analysis for this purpose.[12] The most commonly used technique is CD3 /CD8 staining using immunohistochemistry, but this technique has theoretical flaws, as discussed in chapter 5a. In order to test this theory, in chapter 5b immunohistochemistry was compared to flow cytometric analysis in its ability to correctly identify this premalignant IEL population, and whether the use of this test would influence diagnostic outcomes in clinical practice. In RCD type 2 patients with a dominant aberrant IEL population (>50% of total IELs) immunohistochemistry performed well, but when the aberrant IEL population was only moderately increased (> 20% < 50% of total IELs) half of the patients were erroneously classified as RCD type 1 instead of RCD type 2. It should be noted that once the threshold of an abnormally high percentage of aberrant IEL is surpassed the risk to develop an EATL is increased regardless the exact height of this percentage.[10] Taken together, our data shows a limited sensitivity of immunohistochemistry to adequately identify RCD type 2 patients, which leads to the misclassification of patients and an undesirable delay in onset of treatment. Based on this data we propose that at time of RCD diagnosis all patients should undergo flow cytometric analysis of IELs.

Chapter 6. ***Serum parameters in the spectrum of coeliac disease: beyond standard antibody testing – a cohort study***

So far differentiation between RCD type 2 and other benign forms of CD can only be made with intestinal biopsies collected during upper endoscopy. Therefore we investigated whether levels of various cytokines in peripheral blood could differentiate between various forms of (complicated) CD. In addition to biochemical parameters such as C-reactive protein (CRP), and leukocyte count we determined serum levels of proinflammatory cytokines Il-6, IL-8, IL17a and IL-22, T-cell activation factors soluble (s)CD25 (IL2R-α) and sCD27, T-cell dysregulation factor sCTLA-4, that was previously shown to be up-regulated in various autoimmune disease, and a cytotoxic T-cell parameter granzyme B, and sMICA, previously shown to be associated with the presence of epithelial stress and malignancies. Statistical correction for multiple testing was performed. Our results underline that both RCD type 1 and RCD type 2 are characterized by an inflammatory disease status that shows resemblance to that observed in active CD (not on GFD). RCD type 2 however showed higher levels of IL-6 and granzyme B than patients with active CD. In contrast to IL-6 other proinflammatory parameters (e.g. CRP) did not differ between these groups. Receiver operator curve (ROC) unfortunately showed poor diagnostic characteristics, so that the value of IL-6 and granzyme B in clinical practice appears to be limited. Also, no distinctive levels of cytokine expression were found in the serum of patients with EATL.

Chapter 7. ***Antibody titers against food antigens decrease upon a gluten-free diet, but are not useful for the follow-up of (refractory) coeliac disease***

In this chapter a novel surrogate serological marker for histological recovery in RCD patient was evaluated. Anti-tissue transglutaminase IgA antibodies are used as indicator of mucosal recovery in patients with uncomplicated CD but these antibodies are of no use in RCD as patients adhere to a GFD. Follow-up of the healing of the intestinal mucosa is currently only feasible in these patients by means of an upper gastrointestinal endoscopy during which duodenal biopsies are collected. We hypothesized that due to loss of mucosal integrity antibody formation against common food antigens such as bovine serum albumin would increase. Indeed, titers of antibody titers against bovine serum albumin were higher in patients with CD and RCD as compared to healthy controls. The antibody titers did nonetheless not correlate well with the level of villous atrophy nor with mucosal recovery over time. Therefore, there currently is no clinical use for anti-bovine serum albumin antibodies in RCD.

Chapter 8. ***Origin and immunophenotype of aberrant IEL in RCDII patients***

This chapter focused on the hallmark of RCD type 2, the aberrant IEL, by studying the origin and phenotype of aberrant IELs that are thought to be responsible for the ongoing intestinal inflammation and are at risk to progress to an EATL. Understanding better where these cells originate from and how these cells develop will allow more specific treatment and hopefully better outcomes in the future. Aberrant IELs are characterized by the lack of expression of the TCR-CD3 complex on the cell surface, yet these cells do contain cytoplasmatic CD3 and display TCR rearrangements. Some have suggested that the TCR-CD3 complex is internalized due to overstimulation, implying that these cells originate from mature T-cells.[13] Alternatively, it has been hypothesized that a small unique $CD3^-CD7^+$ IEL population considered as NK/T-cell precursors that was observed in intestine of healthy individuals could be the physiological counterpart of aberrant IELs.[14,15] We studied TCR rearrangement patterns of the gamma, delta and beta chain in DNA isolated from duodenal biopsies of 18 patients. The results were remarkable heterogenic and four patterns could be distinguished. Notably, the only patients who developed an EATL were the three patients in the fourth group which was characterized by a mature stage of TCR(B) development. Considering the small numbers care must be taken with interpretation, but it might be the first clue in identifying more accurately which RCD type 2 patients develop an EATL. More recent data further supports a prognostic role for TCRB gene rearrangement patterns as an association was revealed between containing a high frequency of a dominant TCRB clone, that was determined using high throughput sequencing of TCRB gene rearrangement patterns, with the progression of RCD type 2 to EATL.[16] From our data we concluded that aberrant IELs, for the majority, originate from developing precursor T-lymphocytes but derail during different stages of maturation. The concept of the physiological counterparts of aberrant IELs has been further explored since then and four subsets of lineage negative $CD3^-CD7^+_{ic}CD3^+$ have been identified based on expression of CD127 and CD56 in both healthy controls and CD patients.[17] The massive expansion of aberrant IELs found in RCD type 2 appears to be the result of proliferation from both lineage negative $(Lin^-)CD127^-$ and Lin^-CD127^+ innate IELs.[17] The Lin^-CD127^- innate IELs in RCDII patients already seem defective, as these cells are able to give rise to both NK-cells and T-cells in CD patients, while when harvested from RCD type 2 patients these cells are unable to do so, most likely as a result of chromosomal aberrations found in these premalignant cells.[18]

Chapter 9. ***Differential IL-13 production by small intestinal leukocytes in active coeliac disease versus refractory coeliac disease***

In this chapter we assessed whether the local cytokine profile produced by mucosal intestinal cells from RCD type 2 patients would differ from the cytokine profile of CD patients. We hypothesized that this would be the result of comparing a gluten-mediated inflammatory response in CD, to an inflammatory response independent from gluten which is characteristic for RCD. CD patients with active disease while not yet adhering to a GFD as well as CD patients in remission upon a GFD were included. Furthermore, only patients with RCD type 2 were selected, because they contain, in contrast to RCD type 1, a distinctive IEL population with a unique T-cell

repertoire that has shown to be driven by gluten independent factors.[16] Cells from the lamina propria were isolated and cultured and stimulated with (PMA, ionomycin). No differences were observed between cells harvested from patients with active CD and RCD type 2 with regards to secretion of IFN-γ, TNF-α, IL-17A, IL-5 and IL-10. Only the levels of IL-13 differed between the groups in an unanticipated manner. Levels of IL-13 were higher in patients with RCD type 2 when compared to CD patients with active disease. IL-13 is known to play an important role in gut defense and inflammation, and is upregulated in ulcerative colitis, a chronic inflammatory disease of the colon.[19] IL-13 has shown to have direct cytotoxic effects on epithelial cells, and is produced by NK-cells as part of an innate response, which could be applicable in RCD type 2 where antigenic stimulation is lacking.[19] Somewhat confusing however is that levels of IL-13 were also higher in CD patients in remission when compared to CD patients with active disease, with the IL-13 levels of the former being similar to those found in RCD type 2. Based on our data we can only conclude that by the methods we used the immune response in RCD type 2 and active CD is surprisingly similar, with the exception of IL-13 production.

Chapter 10. *Genetic variations in interleukin 12 related genes in autoimmune disease*

In the era of genome wide-association studies (GWAS) large collaborations have brought us enormous amounts of data by identifying genetic polymorphisms that are associated with complex, multifactorial autoimmune diseases. Now the task awaits to elucidate what those genetic polymorphisms do, to slightly increase (or decrease) the chance to develop such a disease. GWAS have identified over 40 polymorphisms in CD, and in chapter 10 and 11 we have focused on a polymorphism that shows the strongest association with CD, excluding the HLA-system, that is located near the IL12A gene. The IL-12 cytokine family currently consists of heterodimeric cytokines, namely, IL-12, IL-23, IL-27 and IL-35. The IL12A gene codes for the IL-12p35 subunit, that together with IL-12p40 forms IL-12 cytokine, but can alternatively form a heterodimer with EBI3 to form the IL-35 cytokine. The IL-12 cytokine family exerts varying effects in the immune response, so to better understand the genetic associations we first focused on associations of all IL-12 related genes with immune-mediated diseases. Instead of reviewing the different genetic risk factors associated with a particular disease we reviewed the associated diseases with a specific gene cluster. By doing so we found that autoimmune diseases cluster in two groups. Remarkably, the diseases within these groups mirror to a certain extent the known clinical relationship between these diseases. The first group includes T helper 17 / T helper 1 pathway and includes ulcerative colitis, Crohn's disease, psoriasis, ankylosing spondylitis and rheumatoid arthritis. The second group encompasses the T helper 1 / IL-35 pathway and consists of primary biliary cirrhosis, multiple sclerosis, autoimmune thyroid disease and coeliac disease. In general it seems that the IL-12 cytokine family represents a key player in the immune response and genetic polymorphisms that affect its function will likely result in some alteration in the immune response. Nevertheless, several single nucleotide polymorphisms (SNP's) are associated with more than one autoimmune disease. To complicate matters the SNP involved may exert a different effect on their respective trait, meaning it can increase suscep-

tibility for one disease whereas it is protective for the other. The next question is how these polymorphisms alter the transcription and translation of the gene. In agreement with SNP's in other gene regions that are associated with autoimmune disease, only a small minority is located in a coding region (exon) where it alters the protein amino acid sequence and thereby possibly its biologic function.(30) The majority of SNP's are in fact located in introns (≈45%) or in-between genes (≈43%), and these non-coding may alter gene transcription in ways that are more complex to reveal such as affecting enhancers, microRNA's, or long-range transcription regulation.(31) The major challenge now is to elucidate the role of these disease associated gene loci in disease pathogenesis and to identify the functional consequences of these variants.

Chapter 11. *Coeliac disease associated SNP rs17810546 is located in a gene silencing region*

In follow-up of our study regarding genetic associations with IL12 and autoimmune disease in chapter 10, here we attempted to unravel the role of one of the SNPs (rs17810546) with the strongest association with coeliac disease that is also associated with multiple sclerosis, primary biliary cirrhosis and lupus erythematosus.[20] This SNP is located on chromosome 3 at 3q25.33, which is an intergenic region between the genes for SCHIP1 and IL12A.[21] First, we found that this SNP is located in distal enhancer sites of a number of immune regulatory cells known to transcribe the IL12A locus: monocytes, macrophages and neutrophils. The location in such regions make it conceivable that a SNP can alter the recognition site of a transcription factor. While the SNP is located in an intronic region of the long non-coding RNA gene IL12A-AS1 we were unable to find expression in duodenal samples from healthy controls nor CD patients. Next we explored the nature of the region and cloned the surrounding 500 base pares around the SNP. When comparing the DNA sequence of an individual homozygous for the G allele to that homozygous for the A allele another SNP rs7610082 was identified. Presence of the cloned region in transcription experiments showed major down-regulatory effect on the expression of the luciferase gene, which suggests that the cloned region contains sequences to which transcription down-regulatory factors can bind. We then tried to identify the transcription factors responsible for the observed downregulation. Using phylogenetic foot printing we selected four candidate transcription factors, that were subsequently silenced using microRNA (mRNA). None was however able to reverse the down regulatory effect of the cloned region, which means that other, currently unidentified, transcription factors are involved that are involved. Next we investigated whether the presence of the SNP influenced the expression of the IL12A gene in CD. The variant is located in between two genes, IL12A en SCHIP1 but we opted to focus on the IL12A gene because of its prominent role in the immune system. We found that expression of IL12A was strongly upregulated in duodenal biopsies taken from patients with active CD. Only EBI3 was abundantly present, while other candidate binding partners, e.g. IL12B, IL12-p27 were absent. Expression levels of IL12A and EBI3 also correlated well which is suggestive that IL-35 is involved in CD. We also correlated IL12A expression according to genotype which showed a trend towards higher expression in the presence of the risk (G) allele yet did not reach statistical significance which is probably due to the low frequency of the risk allele in

the European population. In conclusion we present evidence that SNP rs17810546 is located in an expression regulatory region and influences expression in a genotype dependent fashion. Furthermore, our data suggests that IL-35 is upregulated in the small intestinal mucosa in CD patients, where it probably exerts proinflammatory effects.

FUTURE PERSPECTIVE

This thesis focused on immune-mediated enteropathies which in general are rather uncommon diseases which limits research possibilities. To gain knowledge and improve treatment outcomes it is of importance to centralize care for these patients in specialized centers. By doing so, differences and overlap between the various immune-mediated enteropathies will become more clear, and it will shed light on the prognosis and optimal treatment of these patients. To further increase expertise and numbers of patients the specialized centers will in their turn increasingly look for international collaborations. The benefits of these collaborations are nicely illustrated by a recent multicenter observational study by in which characteristics of patients with RCD were collected in 7 specialized centers across the world which resulted in a predictive survival model.[22] In addition, an monoclonal antibody that antagonizes IL-15 was evaluated in RCD type 2 patients by means of a large international multicenter trial.[23]

Future research will focus on unravelling the role of the various cell populations involved in the inflammatory process. Studying pathological aberrant intraepithelial lymphocytes has led to the identification of their physiological counterpart of these cells in humans. Revealing more of the numerous cell populations present in the small intestine will provide more insight in the functioning of the mucosal immune system, but also aid in the understanding of immune-mediated enteropathies. For example, in CD numbers of γδ-IELs are increased at time of diagnosis when inflammation is ongoing, but these remain present in high numbers also when the inflammation has faded away after initiation of a GFD, which suggests their role might shift depending in signals from the microenvironment. In common variable immunodeficiency numbers of γδ-IELs may also be increased and insights in the role of γδ-IELs in CD could be relevant for this disease a well, or vica versa. In RCD type 2 it is obvious that the function and stimuli of aberrant IELs have to be further delineated. Recent research showed that these cells, in addition to IL-15, might also proliferate under influence of TNF, IL-2 and IL-21 which means that blocking IL-15 signaling only, might not be sufficient. The recently published data of an anti-IL15 monoclonal indeed showed no effect on reducing numbers of aberrant IEL, but did have a positive effect on diarrhea in RCD type 2 patients.[23] When we would be able to predict more accurately which patients with RCD type 2 will develop an EATL this will allow us to treat those patients even more aggressively, while in the others treatment that will maintain control of their malabsorption related symptoms suffices. To this end studies should further focus on the state of development of the aberrant IEL population and prospectively examine whether this can indeed serve as a prognostic marker.

The association of HLA-DQ2.5 with olmesartan-associated enteropathy and perhaps AIE could be of interest. HLA-DQ 2.5 is strongly associated with CD where it plays a crucial role by presenting gliadin peptides on antigen-presenting cells to gluten reactive T-cells. Recent data also suggests a role for HLA-DQ 2.5 in non-CD immune-mediated disease which could be a first clue in revealing part of the genetic susceptibility in these patients that are commonly affected by autoimmune diseases. Our data suggest that a similar association might hold true for HLA-DQ 2.5 and AIE. The resemblance between AIE and OAE based on histology and with regard to the

presence of anti-enterocyte antibodies in both diseases suggest at least a shared pathogenesis, and a mutual HLA-genotype as risk factor for developing disease further strengthens this theory. The most striking finding in our AIE cohort was that a quarter of patients that had been on long-term immunosuppressive therapy eventually were in long-term clinical remission without the use of any drugs. This implies that this immune-mediated enteropathy might be cured after the pro- and anti-inflammatory equilibrium has been restored. This justifies to look into factors (e.g. medication, viruses) that temporarily put the inflammatory system off balance but do not endure when treated adequately. It also will be interesting to follow-up on these patients and see if the disease recurs later in life. For the unfortunate AIE patients refractory to multiple treatment regimens newer therapies will be studied, such as vedoluzimab an integrin-inhibitor that prevents leukocytes to migrate towards the small intestinal mucosa. In severe, multi-therapy refractory patients treatment with autologous stem cell transplantation in eligible cases will be considered as it has shown promise in one patient.

After the enormous efforts of genome-wide association studies to identify polymorphisms responsible for the genetic susceptibility in immune-mediated diseases, including some of the immune-mediated enteropathies, future research now has to unravel the functional consequences of these genetic variations. When the effect of a certain polymorphism is discovered this will unequivocally lead to new insights in the disease pathogenesis. Yet, revealing the effects that polymorphisms exert is rather complicated as immunogenetic processes are complex and are tightly regulated. Therefore, the success of such studies depends on performing experiments in the tissue or cell on which the polymorphism exerts its effect. To increase difficulty in the correct selection of the so called effector cells, about half of all reported disease associated polymorphisms are actually not located inside a gene. Instead, they are located in between two genes which can be thousands of base pairs away from the closest gene. Thus, to discover what exactly the polymorphism influences will be a challenging but valuable task.

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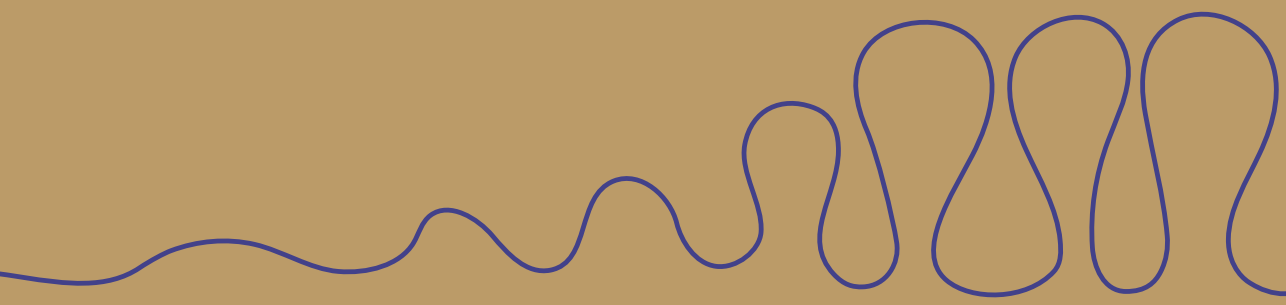

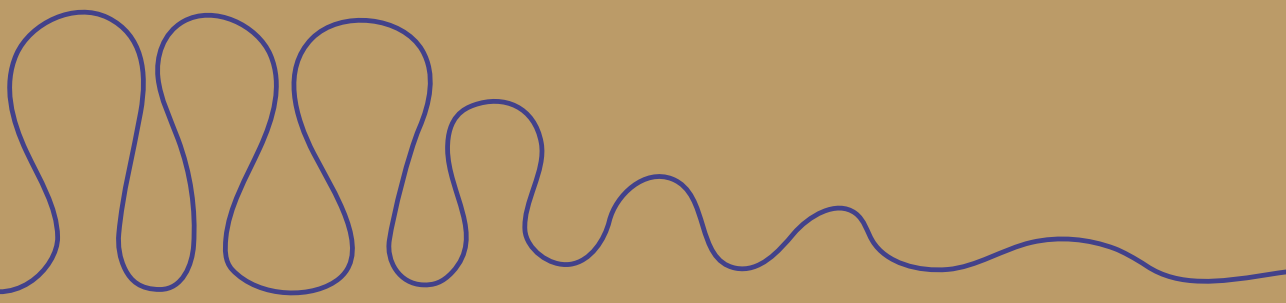

NEDERLANDSE SAMENVATTING

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Introductie

Imuungemedieerde enteropathien zijn aandoeningen van de dunne darm die door een ongepaste en schadelijke (niet-infectieuze) immunologische reactie wordt veroorzaakt. Hierdoor ontstaat schade aan de darmvlokken waardoor de dunne darm zijn taak, absorptie van nutriënten en mineralen, niet meer adequaat kan uitvoeren. Patiënten met een enteropathie ervaren (ernstige) diarree, gewichtsverlies en vitamine deficiënties. In ernstige gevallen moeten patiënten worden opgenomen in het ziekenhuis voor intraveneuze vochtsuppletie en parenterale voeding, en kan soms tot de dood leiden.

In **Deel I** van deze thesis wordt het spectrum van immuungemedieerde enteropathien beschreven. In ***hoofdstuk 1*** wordt een overzicht gegeven van immuungemedieerde enteropathien. Van deze aandoeningen is coeliakie de vaakst voorkomende en tevens ook meest bekende. Andere aandoeningen zijn auto-immune enteropathie, gewone variabele immunodeficiëntie, olmesartan-geassocieerde enteropathie, de ziekte van Crohn, eosinofiele gastroenteritis en graft-versus-host ziekte. De ongepaste immunologische reactie kan worden uitgelokt door gluten (coeliakie), olmesartan (olmesartan-gerelateerde enteropathie) of stamceltransplantatie (graft-versus-host ziekte) in genetisch gevoelige patiënten, hoewel er bij de andere aandoeningen (auto-immune enteropathie, indolent lymfoom) vooralsnog geen uitlokkende factoren bekend zijn. In de klinische praktijk blijkt het soms lastig om te differentiëren tussen deze aandoeningen omdat er overlap is tussen de verschillende ziektebeelden. Dit is met name relevant bij patiënten met coeliakie waarbij een klinische en histologische reactie op het glutenvrij dieet ontbreekt, al dan niet na een initiële goede reactie op het glutenvrij dieet. Bij deze patiënten dient nagegaan te worden of de diagnose coeliakie juist gesteld is en of zij dieettrouw zijn, en tevens moet het bestaan van andere enteropathien onderzocht worden. Indien de diagnose coeliakie bevestigd wordt en er worden geen andere enteropathien ontdekt, dan wordt een patiënt gediagnosticeerd met een refractaire coeliakie (RCD). Er wordt onderscheid gemaakt tussen een type 1 en type 2, waarbij er bij RCD type 2 premaligne monoclonale intraepitheliale lymfocyten (IEL) aanwezig zijn met een afwijkend fenotype, terwijl deze cellen ontbreken bij RCD type 1. Ongeveer de helft van de RCD type 2 patiënten ontwikkelt een enteropathie-geassocieerd T-cel lymfoom, wat een zeer sombere prognose heeft. Daarnaast gaat RCD type 2 gepaard met ernstige diarree en malabsorptie waardoor de 5-jaars overleving van deze patiënten met 58% drastisch verlaagd is. Derhalve is het zeer belangrijk om vroegtijdig de juiste diagnose te stellen zodat een eventueel (agressieve) behandeling met chemotherapie en eventueel autologe stamceltransplantatie kan worden geïnitieerd.

In ***hoofdstuk 2*** beschrijven we 106 patiënten die in de periode 2006-2012 naar ons centrum zijn verwezen in verband met verdenking op refractaire coeliakie. Hierbij is opvallend dat er bij meer dan 20% van de patiënten nog aantoonbare dieetfouten werden gemaakt. Bij een derde van de patiënten werd een darmaandoening – anders dan (refractaire) coeliakie –

geïdentificeerd. Daarnaast blijkt er een groep patiënten met een langzaam histologisch herstel zonder klachten of deficiënties, die onderscheiden worden van patiënten met een langzaam of ontbreken van histologisch herstel die wel klachten van malabsorptie en/of deficiënties hebben en daardoor als refractaire coeliakie worden geclassificeerd. Dit betreft meestal RCD type 1 en deze patiënten hebben wel een behandelindicatie met (lokaal) immunosuppressieve middelen. Uiteindelijk werd in nog geen kwart van alle patiënten refractaire coeliakie vastgesteld, waarvan iets vaker (13%) RCD type 1 dan RCD type 2 (10%). Om een beeld te krijgen van de prevalentie van RCD in Nederland, werden alle Maag-, Darm-, en Leverafdelingen in Nederland gecontacteerd. Dit toonde dat hoewel RCD type 2 een ernstige ziekte is de prevalentie hiervan ook in Nederland erg laag is.

In ***hoofdstuk 3*** beschrijven we 13 patiënten waarbij op volwassen leeftijd een autoimmune enteropathie (AIE) is vastgesteld en dit is de op één na grootste serie beschreven in de wereld. Onze data bevestigt dat AIE een ernstige aandoening is waarbij meer dan de helft van de patiënten moest worden opgenomen om tekort aan vocht en kalium intraveneus te suppleren in verband met ernstige diarree. Ook bleek parenterale voeding bij meer dan twee derde van de patiënten nodig. Histologische afwijkingen bestonden voornamelijk uit actieve chronische ontsteking met apoptotische lichamen en deficiënties van goblet en Paneth cellen. IgA en/of IgG anti-enterocyt antistoffen (AEA) waren aanwezig bij 80% van de AIE-patiënten, maar correleerden niet met de mate van vlokatrofie of klinische presentatie. We hebben AEA's ook getest in andere enteropathien waarbij deze ook werden geïdentificeerd, wat suggereert dat het een secundair fenomeen betreft als gevolg van T-cel gemedieerde weefselschade waardoor (auto)antigenen vrijkomen, en dientengevolge soms (auto)antistoffen worden geproduceerd. Bijna de helft van de patiënten was reeds gediagnosticeerd met een andere auto-immuun aandoening, en bij 85% van de patiënten werden systemische auto-immuun antistoffen gevonden, wat aanleg voor auto-immuniteit suggereert. Voor zover dat betrouwbaar vast te stellen is in een dergelijke kleine groep werd er wel opvallend vaak (79%) aanwezigheid van het HLA-DQ 2.5 genotype gezien.

Steroïden bleken in 62% van de patiënten effectief als inductie-remissie behandeling. Als onderhoudsbehandeling bleken thiopurines en budesonide effectief. In het geval dat steroïden faalden, werden verschillende immunosuppressieve medicamenten geprobeerd, allen helaas met wisselend effect. Eén patiënte was refractair voor meerdere medicamenten waarna zij een autologe stamceltransplantatie onderging en sindsdien is zij in remissie. Drie andere patiënten zijn ook in langdurige klinische remissie (3-7 jaar) zonder het gebruik van medicatie, nadat zij gedurende langere tijd behandeld werden met immunosuppressieve medicatie. Hoewel AIE zeer ernstig kan verlopen en bij drie patiënten (23%) door onbehandelbare malabsorptie ook tot de dood heeft geleid, lijken er ook 4 patiënten (31%) 'genezen' van AIE.

In ***hoofdstuk 4*** beschrijven we een RCD type 1 patiënte die uit een uitzonderlijk grote, monoclonale populatie γδ-IELs in de dunne darm een extra-intestinaal T-cel lymfoom ontwikkelde. Normaliter ontwikkelen patiënten met RCDII, en niet met RCDI, een enteropathie-geassocieerd T-cel lymfoom waarvan gedacht wordt dat deze ontstaat uit de eerder genoemde aberrante IELs. Derhalve is het niet verrassend dat het lymfoom bij deze patiënte

unieke fenotypische en chromosomale kenmerken vertoont in vergelijking met de lymfomen die worden gezien bij RCD type 2. Deze bevinding toont dat intestinale lymfomen uit verschillende voorloper cellen kunnen ontstaan, en onderstrepen de noodzaak voor een nieuwer, completer classificatiesysteem voor enteropathie-geassocieerde T-cel lymfomen.

In **Deel II** zijn bestaande en nieuw ontwikkelde diagnostische testen geëvalueerd om de diagnosestelling alsmede het vervolg van RCD patiënten te verbeteren. In ***hoofdstuk 5*** worden technieken, die worden gebruikt om op basis van fenotype de premaligne IEL in de dunne darm te identificeren, met elkaar vergeleken. Immuunhistochemie met CD3 en CD8 kleuringen op duodenumbiopten wordt in de klinische praktijk het meest gebruikt en wordt vergeleken met de meer nauwkeurige flow cytometrische analyse van levende IELs. Hieruit blijkt dat de immunohistochemische bepaling adequaat 'grote' (indien aberrante IELs > 50% van totaal IELs zijn) aberrante IEL populaties kan identificeren, maar dat bij relatief kleinere populaties aberrante IELs (>20 <50% van totaal IELs) deze techniek slechts de helft correct identificeert. Van belang is dat ook deze kleinere populaties eenzelfde, hoog risico op EATL ontwikkeling hebben. Derhalve is voor differentiatie van RCD type 2 van RCD type 1 in de klinische praktijk immunohistochemische inferieur in vergelijking met flow cytometrische analyse.

Onderscheid tussen RCD type 2 en ongecompliceerde vormen van coeliakie kan tot nu toe alleen gemaakt worden door analyse van duodenumbiopten die tijdens een gastroduodenoscopie verkregen zijn. Daarom hebben we in ***hoofdstuk 6*** onderzocht of door middel van expressie van verschillende ontstekingseiwitten in het perifere bloed onderscheid kon worden gemaakt tussen de verschillende vormen van (gecompliceerde) coeliakie. In patiënten met RCD type 2 bleek expressie van het pro inflammatoire cytokine IL-6 en ook van granzyme B verhoogd, maar bleken niet van waarde voor de klinische praktijk. In ***hoofdstuk 7*** werd geëvalueerd of er een serologische marker voor histologisch herstel konden worden ontwikkeld. Waar bij patiënten met ongecompliceerde coeliakie anti-tissue transglutaminase antistoffen geassocieerd zijn met histologisch herstel, is dit bij patiënten bij RCD per definitie niet het geval. De enige manier naast een klinische inschatting om herstel van patiënten te evalueren is een (belastende) gastroduodenoscopie met duodenumbiopten. We testten de hypothese dat de vorming van antistoffen tegen voedselantigen zou toenemen bij verminderde intestinale integriteit door mucosale schade. Hoewel de titers van antistoffen tegen voedselantigenen inderdaad hoger waren bij patiënten met coeliakie en RCD in vergelijking met gezonde controles, bleek de hoogte van de antistoffen niet goed te correleren met de mate van vlokatrofie en herstel hiervan.

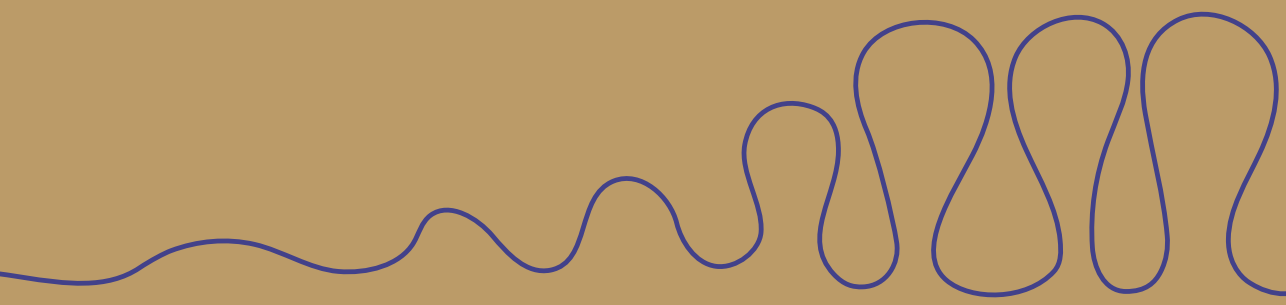
In **Deel III** werd aandacht geschonken aan de immunopathogenese van (refractaire) coeliakie. In ***hoofdstuk 8*** werd onderzocht uit welke voorlopercellen de premaligne, aberrante IELs ontstaan door deze cel populatie fenotypisch te karakteriseren en het T-cel receptor (TCR) herschikkingspatroon te analyseren. De aberrante IELs toonde naast T-cel kenmerken fenotypisch ook NK cel eigenschappen. Aberrante IEL populaties waren opvallend heterogeen in welke fase van de TCR ontwikkeling ze waren ontspoord. Op basis van de mate van

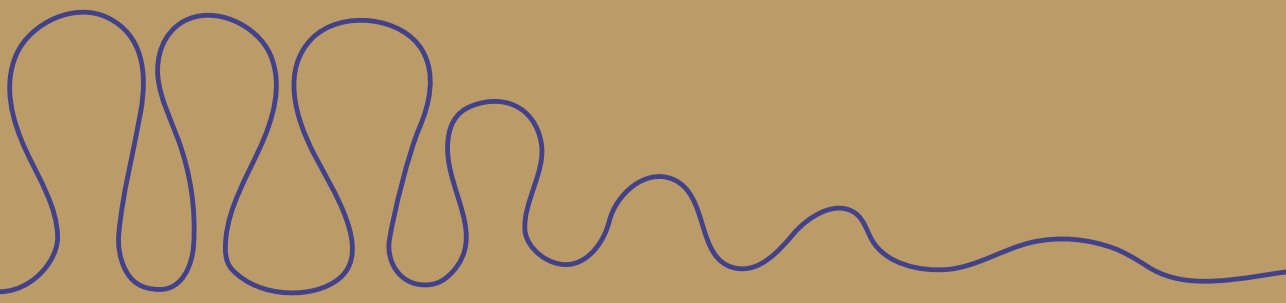
ontwikkeling konden we vier groepen onderscheiden. Opvallend was dat alleen (alle) drie de patiënten uit de laatste groep, waarbij de TCR het meest ontwikkeld was later evolueerde tot een enteropathie-geassocieerd T-cel lymfoom. In ***hoofdstuk 9*** hebben we het cytokineprofiel in de dunne darm vergeleken tussen patiënten met RCD type 2 en coeliakie. We hypothetiseerden dat een glutenonafhankelijke inflammatie zoals bij RCD type 2 zou verschillen van een gluten-gedreven inflammatie bij coeliakie. Het cytokineprofiel van uit duodenumbiopten geïsoleerde leukocyten van RCD type 2 patiënten was opvallend overeenkomstig met dat van patiënten met coeliakie, waarbij alleen een significant verschil in IL-13 productie werd gezien.

Een van de sterkste genetische associaties van coeliakie is met een polymorfisme gelegen nabij het *IL12A* gen. De interleukine 12 (IL-12) cytokine familie bestaat uit 4 verschillende cytokinen die alle bestaan uit heterodimeren. Sub units worden gedeeld binnen de IL-12 familie. Zo bestaat het pro-inflammatoire cytokine IL-12 uit IL-12p40 en IL-12p35 die respectievelijk worden gecodeerd door de genen *IL12A* en *IL12B*. Het eiwit IL-12p40 vormt samen met IL-23p19 een ander pro inflammatoire cytokine, namelijk IL-23. IL-12p35 kan op zijn beurt ook aan een ander eiwit binden, namelijk EBI3, en zo het cytokine IL-35 vormen. Om meer inzicht te verkrijgen in de rol van genetische associaties in de IL-12 familie met auto-immuunziekten, hebben we in ***hoofdstuk 10*** vanuit deze genen gekeken naar alle associaties die met de verscheidene auto-immuunziekten geïdentificeerd zijn. Hierbij viel op dat auto-immuunziekten clusteren in een tweetal groepen: een groep vertoont associaties met het *IL12A* gen wat betrokkenheid van T-helpercellen 1 en IL-35 impliceert, en de andere groep met *IL12B* en *IL23R* wat beïnvloeding van T-helpercellen 1 en T-helpercellen 17 suggereert. De grote meerderheid van de geïdentificeerde SNP's bleken in intronen (≈45%) of tussen genen in gelegen (≈43%) te zijn, en deze niet-coderende delen beïnvloeden de transcriptie van genen op manieren die een stuk lastiger te achterhalen zijn dan wanneer deze zich in een voor een eiwit coderend exon bevindt. In ***hoofdstuk 11*** hebben we getracht om het effect van SNP rs17810546 te achterhalen omdat deze een van de sterkste (niet-HLA) associaties heeft met coeliakie. Deze SNP bevindt zich in een zogenaamde 'enhancer site' van een aantal immuun regulerende cellen die het IL12A locus aflezen: monocyten, macrofagen en neutrofielen waardoor het voorstelbaar is dat deze SNP de herkenningssite van een transcriptie factor kan veranderen. We hebben 500 baseparen rondom deze SNP gekloneerd en hier transcriptie experimenten mee uitgevoerd die inderdaad onthulde dat deze klonen enorme downregulatie van transcriptie induceren. Vervolgens trachtten we de hiervoor verantwoordelijke transcriptie factor te identificeren. Met behulp van fylogenetische voetafdrukken selecteerde we een viertal transcriptiefactoren die we blokkeerden middels microRNA. Echter, bleek het blokkeren van geen van de factoren het down regulerende effect van de kloon ongedaan te maken, waardoor het op dit moment niet duidelijk is welke transcriptie factor verantwoordelijk is voor dit effect. Hierna hebben we onderzocht of IL12A een rol speelt in coeliakie. Expressie van IL12A in duodenumbiopten van patiënten met coeliakie bleek significant hoger dan in die van controle patiënten. EBI3 bleek de enige bindingspartner die ook in het weefsel aanwezig was (IL12B, IL12-p27 waren niet of amper detecteerbaar), en de hoeveelheid EBI3 correleerde ook met die van IL12A, wat tezamen suggereert dat IL-35 een rol speelt in coeliakie.

PART V

ADDENDUM





DANKWOORD / ACKNOWLEDGEMENTS

DANKWOORD (ACKNOWLEDGEMENTS)

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Beste Rogier, vriend sinds heugenis, dank voor je immer aanwezige steun, goede gesprekken en droge humor. Hoog tijd om jou en het goede leven in Albanië op te zoeken.

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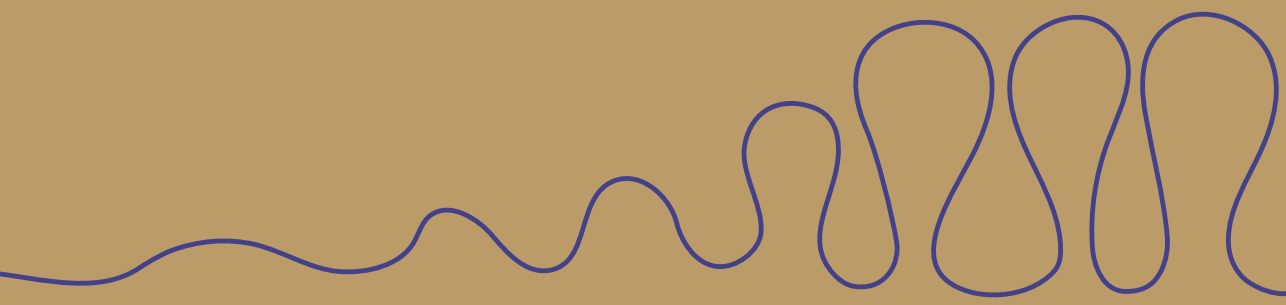
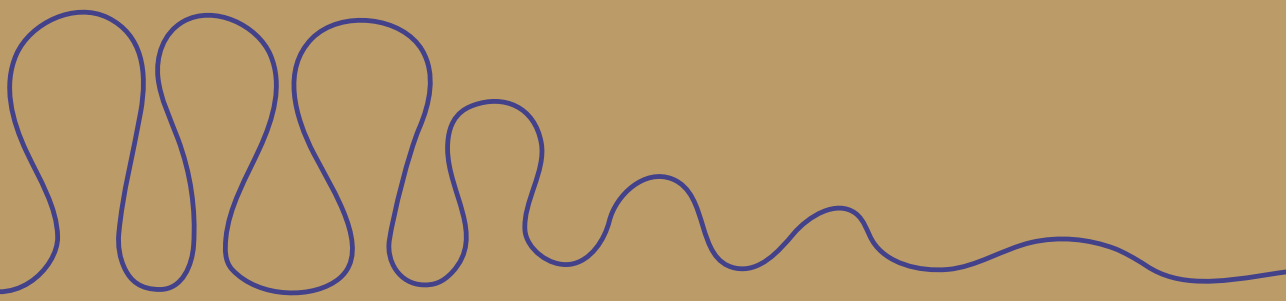
Lieve Ad en Petra. Onvoorwaardelijke liefde en steun heb ik van jullie gehad. Net als de les dat als je iets wilt bereiken je daar elke dag hard voor moet werken. Kenny, lieve broer, wat ben je toch een ontzettend fijne goedzak, jouw behulpzaamheid is oneindig. Wendy, lieve zus, wat fantastisch dat ik oom van Isabel en Emily ben geworden.

Tot slot, mijn lieve Maryam. Vorig jaar mocht ik vol trots jouw verdediging bijwonen. Ik ervaar elke dag jouw levensenergie en dit heeft een aanstekelijke werking. Je bent zelf altijd druk bezig, maar tegelijkertijd ben je er altijd voor mij. Je steun was de laatste jaren grenzeloos en zo belangrijk voor me. Dat ik de pad van het leven met jou aan mijn zijde mag bewandelen voelt elke dag als een verrijking. Ik hou van je en wil nog vele avonturen met je beleven.

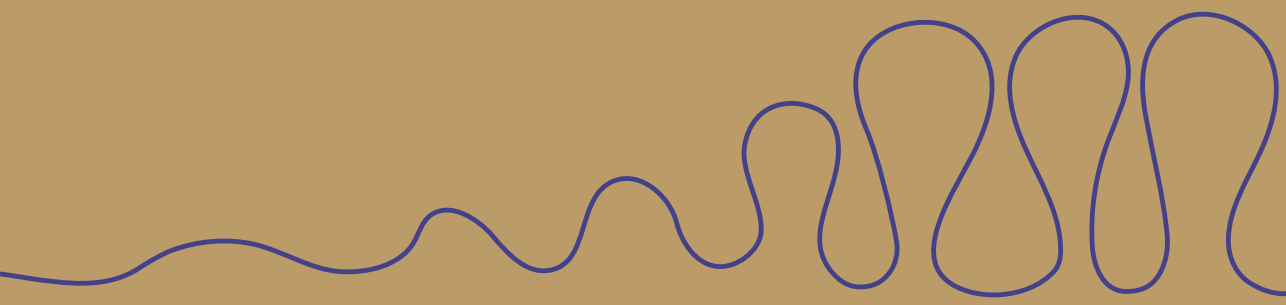


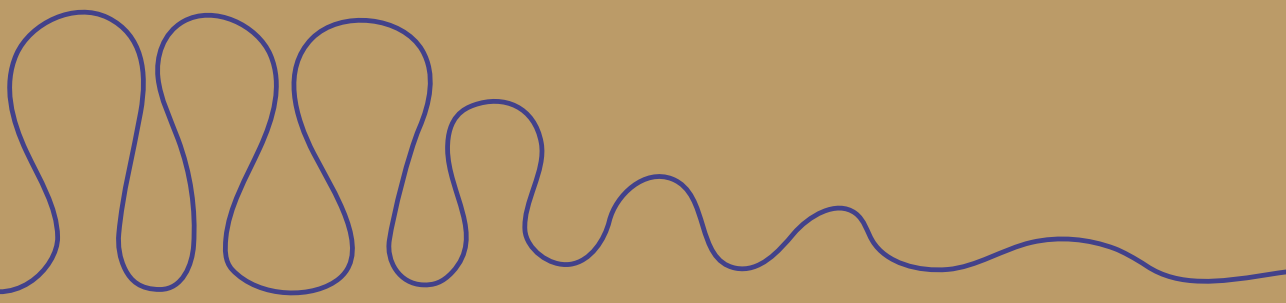


CURRICULUM VITAE

CURRICULUM VITAE

Roderik (Roy) Leonardus Johannes van Wanrooij werd geboren op 30 april 1984 in Oss.
Hij groeide op in het Brabantse Heesch en volgde het tweetalig Voorbereidend Wetenschappelijk Onderwijs (VWO) aan het Maaslandcollege. Na het behalen van de propedeuse Bewegingswetenschappen aan de Vrije Universiteit in Amsterdam, startte hij in 2003 met de studie Geneeskunde aan diezelfde universiteit. Gedurende de studie volgde hij extra-curiculaire klinische stages in The Apostolic Hospital te Banga Bakundu (Kameroen) en Steve Biko Academic Hospital te Pretoria (Zuid-Afrika). Vanaf 2006 was hij werkzaam als student-onderzoeker op de afdeling Maag-, Darm- en Leverziekten (MDL) van het VU medisch centrum (VUmc) onder begeleiding van dr. J.S. Terhaar sive Droste en prof.dr. C.J.J. Mulder.
Roy richtte zich tijdens zijn wetenschappelijke stage naar de rol van het mucosale immuunsysteem bij chronisch darmziekten aan de University of Alberta, Edmonton, Canada. Na het behalen van de artsenbul in 2010 startte hij met promotieonderzoek onder leiding van prof. dr. G. Bouma en prof. dr. C.J.J. Mulder op de afdeling MDL VUmc. De vooropleiding Interne Geneeskunde werd in 2012-2014 gevolgd in het Rode Kruis Ziekenhuis te Beverwijk (opleider Niek Valk), waarna hij de opleiding tot MDL-arts in het Noordwest Ziekenhuis (opleider dr. M. Klemt-Kropp) en het VUmc (opleider dr. M.A.J.M. Jacobs) doorliep en deze afrondde op 30 april 2018.
Sinds 1 mei 2018 is hij werkzaam als MDL-arts in het Amsterdam UMC (afdelingshoofd prof. dr. P. Fockens). Hij woont in Amsterdam samen met zijn vriendin Maryam Soltanipoor.





LIST OF PUBLICATIONS

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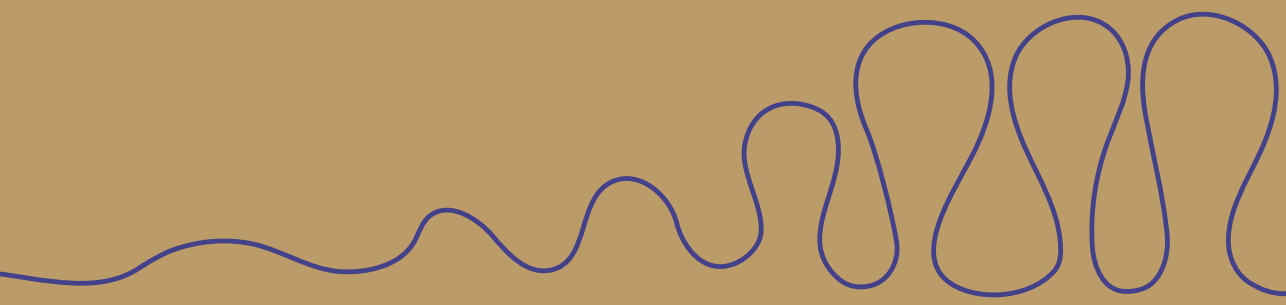

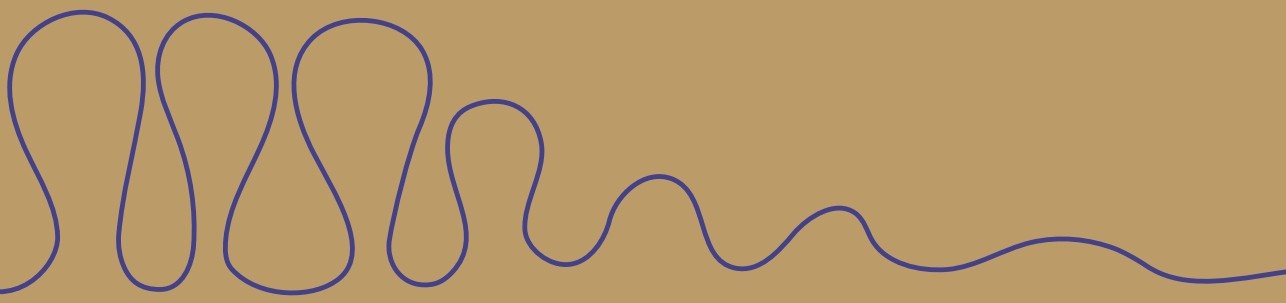

LIST OF ABBREVIATIONS

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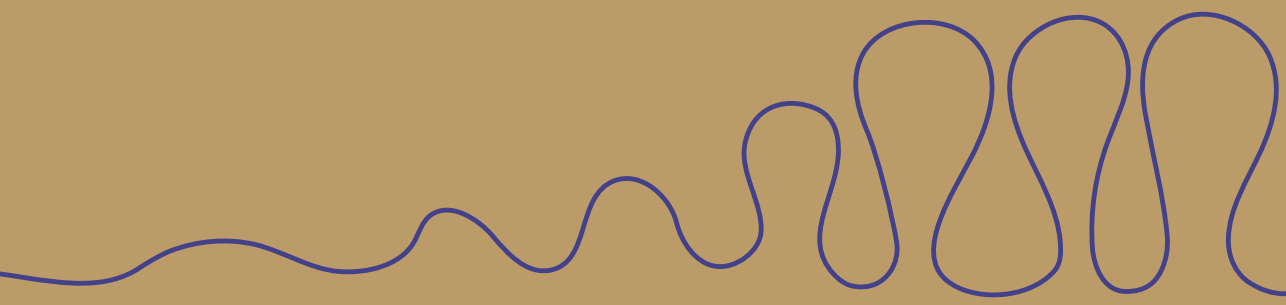
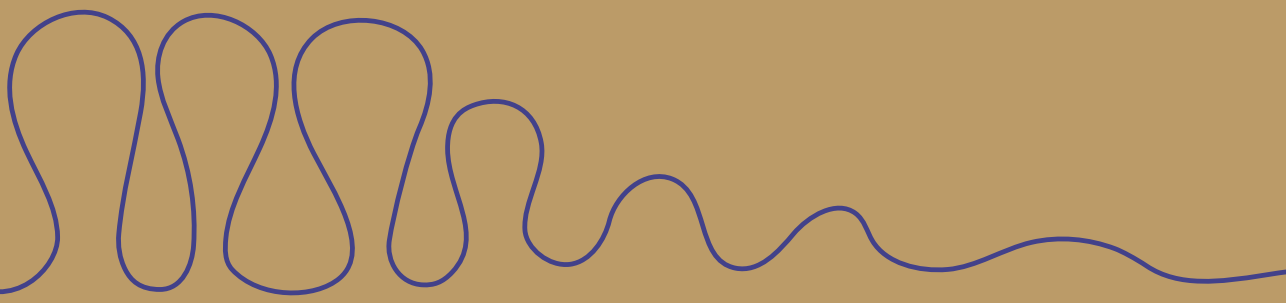
AGCA	anti-goblet cell antibody
AIE	autoimmune enteropathy
AIG	autoimmune gastritis
AEA	anti-enterocyt antibody
CVID	common variable immunodeficiency disorder
CD	coeliac disease
CrD	Crohn's disease
EATL	enteropathy-associated T-cell lymphoma
EMA	anti-endomysium antibodies
EBI3	Epstein Barr induced virus 3
GFD	gluten-free diet
GvHD	graf-versus-host-disease
GWAS	genome wide association studies
HLA	human leukocyte antigen
IFABP	intestinal fatty acid-binding protein
IEL	intraepithelial lymphocyte
IL	interleukin
IME	immune-mediated disease
IPEX	immune dysregulated polyendocrinopathy X-linked
NK	natural killer
OAE	olmesartan-associated enteropathy
PCA	anti-parietal cell antibodies
RCD	refractory coeliac disease
RCDI	refractory coeliac disease type 1
RCDII	refractory coeliac disease type 2
SMA	anti-smooth muscle antibody
SNP	single nucleotide polymorphism
TCR	T-cell receptor
TCRG	T-cell receptor gamma
TGA	anti-tissue transglutaminase antibodies
TNF	tumor necrosis factor
TPO	thyroid peroxidase antibody





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